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THE MEASUREMENT OF THE OXYGEN CONSUMPTION OF *DAPHNIA* BY A MODIFICATION OF THE CARTESIAN DIVER TECHNIQUE

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(With Two Text-figures)

INTRODUCTION

The use of the Cartesian diver as a micromanometer was first described by Linderstrøm-Lang (1937). Boell, Needham & Rogers (1939) adapted the method to the measurement of the respiration of fragments of amphibian embryos and, in this and subsequent papers, many details of technique were worked out (see Needham, Rogers & Shen Shih-Chang, 1939). Boell, with other collaborators, has used the technique to measure the respiration of early mammalian embryos (Boell & Nicholas, 1939), and to estimate the distribution of cholinesterase (Boell & Nachmansohn, 1940; Boell & Shen Shih-Chang, 1944). Further use of the technique in Linderstrøm-Lang's laboratory has included the measurement of the oxygen consumption of tissue cultures (Zamecnik, 1941). A detailed theoretical analysis of the method has been undertaken (Linderstrøm-Lang & Holter, 1942; Linderstrøm-Lang, 1943), and based on this further technical details have been worked out (Holter, 1943). In all these methods the total capacity of the divers has been less than 40 μl., and this capacity has been greatly reduced in further modifications of the technique described by Zeuthen (1943, 1946).*

In order to adapt the method to the measurement of the oxygen consumption of *Daphnia* it was found necessary to design divers capable of accommodating 40–50 μl. of fluid in which four to five *Daphnia* were suspended. Thus it became necessary to increase the total capacity to over 100 μl., creating certain possibilities of error, which have been experimentally investigated, and the results are included in the description of the modified technique.

DESCRIPTION OF THE TECHNIQUE

Briefly to recapitulate the principles of the method, the reaction vessel, or diver, is made of capillary tubing and consists of an open neck, an expanded bulb, and solidified tail which causes the diver to float tail downwards. If the diver is floated in a suitable vessel and the pressure within the vessel altered, fluid can be made to enter or leave the neck, thus altering the buoyancy of the diver so that, by suitably controlling the pressure, the diver can be brought to equilibrium at a determined level. Any change in the volume of gas within the diver will alter the pressure necessary to produce this equilibrium.

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* Woerderman & Raven (1946) refer to a further modification by Ten Cate.

Construction of the divers

The shape and structure of the divers are indicated in Fig. 1, where it will be seen that, in addition to the neck, bulb, and solid tail there is a central cup incorporated in the tail and projecting into the bulb for about two-thirds of the diameter. This central cup contains sodium hydroxide solution for the absorption of carbon dioxide. Although there is some variation in the size of the divers used, the following are approximate average dimensions:

Total capacity	120 μl.
Length of neck	15 mm.
Internal diameter of neck	1·3 mm.
Internal diameter of cup	0·5 mm.

The dimensions are such that the bulb is half filled by 40–50 μl. of fluid.

The divers are made by blowing a bulb 2–3 cm. from the end of a suitable piece of capillary tubing, and inserting a second piece of tubing within the first. A close fit is necessary and the second piece of tubing is made to protrude the required distance into the bulb. The double layer of tubing is then solidified to form the tail, care being taken to obliterate the space between the two layers of tubing. The tail and neck are then cut off at the required lengths. The divers can be cleaned and used repeatedly.

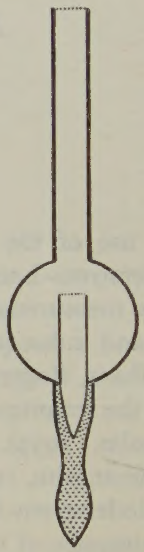

Fig. 1. Structure of divers.

Filling the divers

To introduce the *Daphnia* the divers are completely filled with the suspension medium, which in this case is filtered aquarium water. *Daphnia* are picked up by a platinum loop, introduced into the necks of the divers, and made to enter the bulb by means of a straight platinum wire. Excess fluid is then removed by a capillary pipette leaving about 40–50 μl. fluid in the bulb. Any fluid in the central cup is removed. The internal surface of the neck is then dried with strips of filter-paper and coated with a layer of paraffin wax introduced by a heated wire. Care is taken to see that the coating stops precisely at the junction of neck and bulb and that the amount of wax does not materially reduce the internal diameter of the neck. Next, by means of a waxed capillary pipette, 5% sodium hydroxide solution is introduced into the central cup filling it to within 2 mm. of its opening. It is convenient to colour the sodium hydroxide solution.

The air in the divers is then replaced by oxygen. About 100 c.c. of moist oxygen are passed through the divers. During subsequent manipulations some of the oxygen may be lost by diffusion, but by absorbing the contained gas with alkaline pyrogallol solution it has been shown that the atmosphere in the divers during the actual readings is over 95% oxygen.

In those cases where a reagent is to be added to the fluid in the bulb during the period of observation, this is introduced in solution into the neck to form a column of fluid immediately adjacent to the bulb. The concentration of the reagent should be adjusted so that the required amount is contained in a column of fluid approximately 4 mm. in length. An oil seal is next added consisting of a column of light liquid paraffin about 2 mm. in length and separated from the reagent by about the same distance. Oxygen is again passed into the open portion of the neck, and the terminal 3 mm. filled with flotation fluid (see next section), leaving a gas space between the flotation fluid and the oil seal.

At this stage it is convenient to calculate the amounts of fluid added to the neck by measuring the lengths of the fluid columns, and the internal diameter of the neck, by means of a microscope with a measuring eyepiece. The amount of fluid in the bulb is obtained by weighing the divers at the appropriate stages of the filling process.

Flotation fluid—adjustment of diver weight

Previous investigations (Linderstrøm-Lang & Holter, 1942; Boell *et al.* 1939) have shown that the properties of the flotation fluid have an important effect in limiting the amount of gaseous diffusion that occurs between the flotation fluid and the contents of the diver. In the present method the fluid used is a sodium chloride solution of a specific gravity of 1·1. Divers filled as described are transferred to a beaker filled with this fluid in which they float easily. Fragments of plasticine are dropped into the beaker, picked up by the tail of the diver, and the amount adjusted until the diver just floats in the fluid. This adjustment should be performed at approximately the temperature at which the measurements are to be made and the fluid in the beaker is, therefore, warmed when necessary.

Flotation tubes

When the weight of the divers has been adjusted they are transferred to the flotation tubes which are 15 cm. in length and 3 cm. internal diameter. These are four-fifths filled with the flotation fluid and midway in their length a horizontal circular mark is etched—the flotation level. After introducing the divers—one to each tube—a disk of cork is dropped in. This floats at the surface and prevents contact between the surface of the fluid and the neck of the diver avoiding displacement of fluid in the neck by surface-tension effects. Before the introduction of the divers, the fluid in the tubes is saturated with oxygen.

Water-bath—control of pressure in flotation tubes

Six flotation tubes, each containing a single diver, are suspended vertically in a water-bath with a glass front. The temperature is controlled to within less than 0·1° C. which is adequate if a control diver is used. Each flotation tube is connected to an open water manometer capable of measuring a pressure change of 100 cm.

water, and to an apparatus to vary the pressure in the tubes. This consists of a 20 c.c. syringe with a 5 c.c. syringe as a fine adjustment, the alteration in pressure being transmitted by the air contained in the connecting tubes. Taps are incorporated in the system so that the pressure can be varied and measured in each flotation tube independently (see Fig. 2).

Addition of reagent

The method of transferring the reagent from the neck to the bulb contents is derived from Boell, Koch & Needham (1939). The manometer is shut off by closing tap 13 in Fig. 2 and then sufficient pressure can then be created by the 20 c.c. syringe to drive the reagent over the internal surface of the bulb so that it mixes with the bulb contents. The pressure can be controlled so that none of the oil leaves the neck and is returned to its original position on release of the pressure. Observations with coloured fluids indicate that about 20 min. should be allowed for mixing, but this period is considerably reduced when the bulb contains actively moving *Daphnia*.

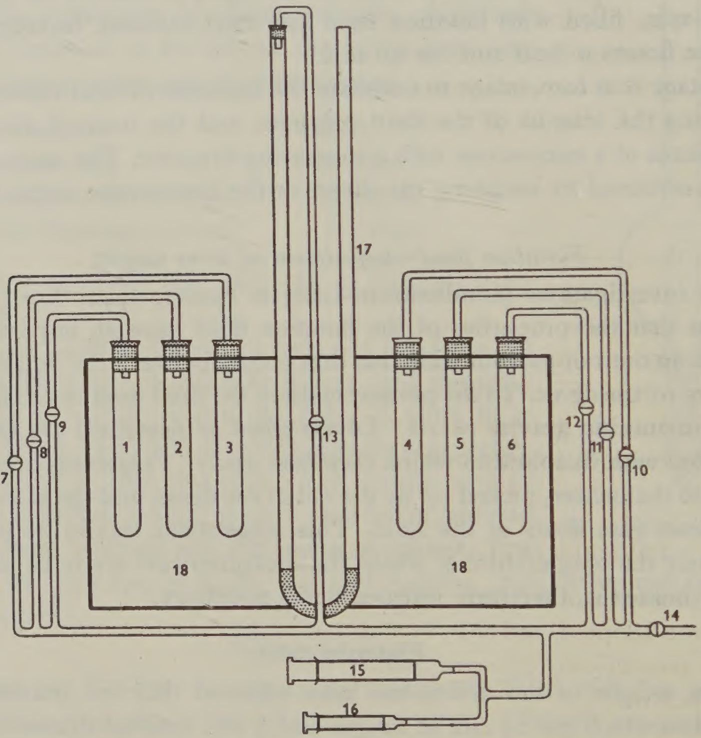

Fig. 2. Arrangement of apparatus. 1–6, flotation tubes; 7–14, taps; 15, 16 syringes; 17, manometers; 18, thermostatically controlled water-bath.

Method of making readings—calculation of results

In between readings it has been found most convenient to decrease the pressure in the flotation tubes so that the divers come to rest in contact with the under, submerged surface of the cork disk. When a reading is to be made, the pressure is increased so that the diver sinks, and by adjusting the pressure, is brought to equilibrium at the flotation level, this being judged by direct vision. Equilibrium is considered to be reached when the diver remains steady for 5–10 sec. The pressure at this equilibrium—the flotation pressure—is then read and the diver returned to the top of the flotation tube. It has been found that the readings can be reproduced to within 1 mm. water.

Each time the diver is brought to equilibrium the volume of the contained gas is brought to a constant volume, so that the diver acts as a constant volume manometer and the relationship between change in volume and change in flotation pressure is given by the equation of Boell *et al.* (1939), which contains a term to allow for the gas dissolved in the fluid within the diver. In the present case the gas is oxygen and the low coefficient of solubility reduces this term to less than 3% of the oxygen uptake. It can, therefore, be ignored in most calculations so that when measurements are made at 27° C. the oxygen uptake is given by

$$V = p\frac{Vg}{A}\frac{273}{300},$$

where V is the amount of oxygen taken up in microlitres, Vg is the volume of the gas space in the diver in microlitres, p is the change in flotation pressure in millimetres water, and A is the atmospheric pressure in millimetres water. Vg is obtained by subtracting the volume of fluid in the diver from the total volume—the latter being obtained by weighing when filled with mercury. The calculations are performed for each experiment, but dimensions of the divers are such that 1 cm. change in flotation pressure corresponds to 0·05–0·08 μl. oxygen.

INVESTIGATION OF THE MODIFIED TECHNIQUE

A comparison with other techniques will show that many precautions suggested have not been adopted, and this, in addition to the increased size of the diver, leads to certain possibilities of error which have been investigated by experiments described below.

Performance of control divers

Here divers have been filled as described, except that the bulb contained a non-reacting fluid so that any changes in the flotation pressure would be due to physical conditions, such as fluctuations in the temperature of the water-bath, changes in atmospheric pressure, and interchange of gas between the contents of the diver and the flotation fluid. Two oxygen-filled control divers were observed at frequent intervals for a period of 5 hr. In one case the flotation fluid was saturated with air, in the other with oxygen. In neither case was there any tendency for the flotation pressure to alter in a constant direction, and the maximum change in the flotation pressure in any one hour was 2 mm. water. Thus it is taken that the fluctuations are due to minor alterations in the physical conditions and that these, in actual experiments, would be adequately controlled by the use of a control diver.

Accuracy of the addition of reagents—accuracy of measurements

The possibility was considered that some of the reagent added to the bulb from the neck might adhere either to the neck or the bulb and not mix with the bulb fluid. To test this N/10 HCl was placed in the bulb and 0·1% sodium bicarbonate added from the neck. In this case there was no sodium hydroxide solution in the central cup, so that the carbon dioxide evolved caused a change in the flotation pressure of the diver. From this the amount of carbon dioxide evolved could be calculated by appropriately modifying the formula given on page 317 so as to include the carbon dioxide dissolved in the fluid in the diver. This was then compared with the amount of carbon dioxide to be expected from the amount of sodium bicarbonate added. The results of five such experiments are given in Table 1. It should be noted that the altered flotation pressure was reached in less than 15 min.

Table 1. *Evolution of carbon dioxide from sodium bicarbonate solution*

Figures in μl. at N.T.P.

Exp.	Calculated	Measured by Cartesian diver	Difference (%)
1	1·62	1·71	5·6
2	1·79	1·86	3·9
3	2·01	1·97	2·0
4	2·35	2·34	0·4
5	2·06	2·05	0·5

Table 2. *Rapidity of absorption of carbon dioxide*

Exp.	Amount of CO_2 evolved (μl.)	Time to regain original flotation pressure ±2 mm. water (min.)
1	3·8	8
2	4·0	12
3	3·7	11
4	4·0	15
5	3·9	10
6	3·9	10
Average	3·9	11

Speed of absorption of carbon dioxide

In the Barcroft technique Dixon & Elliot (1930) have shown that the rate of absorption of carbon dioxide by alkali in a central cup may be inadequate. Since, on a smaller scale, the conditions here are comparable, this point was tested by direct experiment. As in the previous section, sodium bicarbonate solution was added to hydrochloric acid in the bulb, but in this case the cup of the diver contained sodium hydroxide solution. When the sodium bicarbonate was added to the acid there was a slight increase of buoyancy, followed by a return to the original flotation pressure. In Table 2 are set forth the amounts of carbon dioxide evolved in five experiments, and the time required for the divers to return to within 2 mm. of the original flotation pressure. From the results it is seen that an average of

3·9 μl. carbon dioxide was absorbed in an average of 11 min. This amount of carbon dioxide is greater than that evolved in 2 or 3 hr. when the oxygen uptake of *Daphnia* was measured.

Adequacy of oxygen supply

When the bulb contains actively moving *Daphnia* no deficiency of oxygen supply would be expected because the movement of the water fleas keeps them near the surface and imparts an appreciable amount of movement to the fluid. This expectation is borne out by the fact that replacement of the oxygen by air does not diminish the rate of oxygen uptake. However, experiments were designed when the oxygen uptake of paralysed *Daphnia* was to be measured. In this case the *Daphnia* sank to the bottom of the bulb and were separated from the atmosphere of the diver by a layer of fluid 2–3 mm. in depth, through which the oxygen used would have to diffuse.

It was thus necessary to ascertain that this rate of diffusion was adequate to meet the oxygen needs of the paralysed *Daphnia*. The filling conditions of the divers were, therefore, reproduced with a suspension of baker's yeast, the respiratory rate of which was certain to exceed the rate of diffusion of oxygen into the fluid in the bulb of the diver. During the process of filling the divers these were centrifuged to pack the yeast cells at the bottom of the bulb. When measurements of oxygen uptake were made, the results obtained represented, not the respiratory rate of the yeast, but the rate of diffusion of oxygen into the bulb fluid. All divers were tested in this way before being put into use, and none were used unless the rate of diffusion under these conditions was 2 μl./hr. or more. In experiments involving measurements on paralysed *Daphnia* the rates of oxygen uptake were considerably less than this.

Table 3. *Oxygen consumption of normal* Daphnia

Figures in μl./flea/hr.; each diver contained five fleas; measurements made at 27° C.

Diver	Day				
	1	2	3	4	5
1	0·23	0·16	0·40	0·28	0·25
2	0·24	0·16	0·40	0·27	0·27
3	0·25	0·19	0·35	0·26	0·28
4	0·23	0·19	0·36	0·24	0·26
5	0·22	0·17	0·34	0·31	0·28
Average	0·23	0·17	0·37	0·27	0·27

THE OXYGEN CONSUMPTION OF NORMAL *DAPHNIA*

Both *D. pulex* and *D. obtusa* were used. These were gathered from ponds and stored in aquaria until required for use. Each measurement was made on four or five individuals between 1·5 and 2·0 mm. length suspended in 40–50 μl. of fluid in a single diver. No differences were noticed in the two species so they are not distinguished in the results given. Measurements were made at 27° C. and extended over an hour or more, resulting in a change of flotation pressure of more than 150 mm. water. In Table 3 are set forth the results of a series of measurements performed on different days and on different batches of *Daphnia*, each measurement consisting of five divers each containing five *Daphnia*. The results are expressed in μl./flea/hr. It will be seen that although there is a considerable variation in the average results obtained on different days, with different batches of *Daphnia*, the results on any one day are considerably more constant.

DISCUSSION

In order to accommodate the *Daphnia* the size of the divers has been considerably increased as compared with other techniques. Although this allows the divers to be filled without special pipettes there is a decrease in sensitivity. In the present technique a change of 1 cm. water in flotation pressure represents a gas change of 0·05–0·08 μl., whereas in the technique of Boell *et al.* (1939) a similar change of pressure corresponds to 0·003–0·022 μl. and in the smaller diver, as described by Zeuthen (1943), this figure would be still smaller.

In the case of *Daphnia* with a high and variable rate of oxygen consumption, the resulting loss of accuracy is not of consequence, but since the technique could be applied to other material, the degree of accuracy is worthy of consideration. From Table 1 it is seen that measurements of 2 μl. of a change can be measured to within an accuracy of 10%, and although the gas concerned was carbon dioxide, the error in the case of oxygen would not be expected to be any greater. This gas change is represented by about 300 mm. change in the flotation pressure so that the errors in its readings would not be of consequence. This amounts to 1 mm. water in the reading of a single pressure, and gives rise to a possibility of an error of 2 mm. when the difference between two pressures is measured, and this would increase the error by a further 10% when the pressure change was 20 mm. water corresponding to a gas change of approximately 0·12 μl. On the other hand, it can be pointed out that these are maximum estimates which could in all probability be reduced on the basis of further experimental analysis. Further, the technique described is designed to measure the change of oxygen uptake resulting from the addition of reagents without altering the fluid contents of the diver, so that such a change can be measured without introducing the errors due to a calculation of the relationship between the change of flotation pressure and the gaseous change.

If the material used does not undergo gross movement, as is the case in *Daphnia*, the limits imposed by the rate of diffusion of oxygen become important. In the case of non-motile material the present investigations show that, with divers of the type described, there is an upper limit at the rate of 2 μl. of oxygen per hour. Although this would be greater in the case of uniform cell suspensions or with a chemical reaction proceeding uniformly throughout the fluid to the bulb, nevertheless, a specific investigation of the limits of diffusion would be advisable if the experimental conditions were altered.

SUMMARY

1. The Cartesian diver microrespirometer has been adapted to the measurement of the oxygen consumption of *Daphnia*. The modification involves an increase in size of the diver to a total capacity of over 100 μl., and absorption of carbon dioxide by sodium hydroxide in a central cup incorporated in the structure of the diver.

2. The variation in the rate of oxygen consumption of normal *Daphnia* has been measured.

3. Sources of error in the modified technique have been investigated and the possibility of its wider use discussed.

The writer is indebted to Prof. R. J. V. Pulvertaft for facilities provided and for many helpful suggestions.

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THE FLIGHT OF BIRDS

THE FLAPPING CYCLE OF THE PIGEON

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(With Plates 5–8 and 12 Text-figures)

For many years there has been discussion on the details of the wing movements of birds in flight, and many attempts have been made to produce clear cinematograph records of the flapping cycle. Until recently no photographic system has combined a sufficiently short exposure with a rapid repetition rate and a reasonably large field of view.

The development of the Arditron discharge tube has provided a light source which is of very high intensity, and has a flash duration of about 1 μsec. This lamp, though designed for single-flash operation, has been found very suitable for high-speed cinematography.

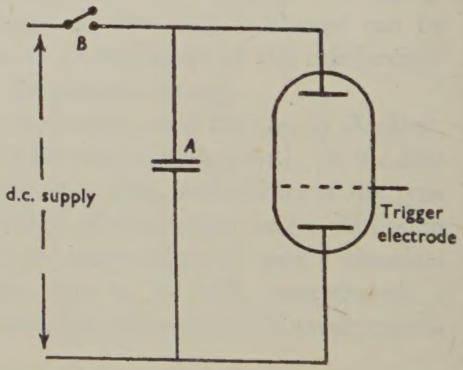
The purpose of this paper is to describe the cinematographic technique which has been developed, and to give a preliminary account of some of the information which has been obtained on the slow flight of the pigeon.

PHOTOGRAPHIC TECHNIQUE

As stated above, the photographic method is based on the use of an Arditron type tube, the characteristics of which determine the limitations of the method and must, therefore, be considered first.

Lamp. This is a glass tube filled with a gas mixture (chiefly argon) and having electrodes at each end which are photo-emissive.

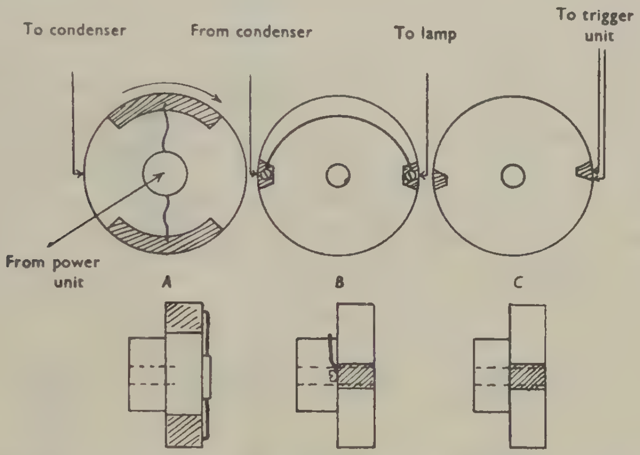
A third electrode near one end is used to start the discharge; this is known as the trigger electrode. The basic circuit is shown in Text-fig. 1. The condenser A is charged from a source of high voltage (about 8000 V.) which is then disconnected. On applying a pulse of high voltage to the trigger electrode the tube becomes conducting, and the condenser is discharged very rapidly through the tube, giving an intense flash of light. In practice for photography by single flashes, as opposed to repetitive flashing for cinema work,



Text-fig. 1. Lamp circuit reduced to its simplest form. The condenser A is charged by the closure of switch B which is then reopened. The lamp is flashed by a voltage pulse applied to the trigger electrode.

the switch *B* is replaced by a resistance of a value which delays the rise of voltage on the condenser, and gives time for the tube to return to a non-conducting state. If this resistance is too small, and the rise of voltage too rapid, the tube flashes spontaneously before the condenser voltage has reached its maximum, thus producing a continuous series of flashes until the power is switched off. For single-flash photography the high resistance and slow recharge is no disadvantage, but for repetition flash work at 50–100 pictures per sec. the necessary conditions are very difficult to satisfy. In the apparatus to be described the resistance-charge method was abandoned and replaced by a rotary switching system linked to the camera; this has given complete satisfaction.

The complete apparatus may conveniently be subdivided into the following sections: switching system, trigger unit, power unit, camera, camera and switch drive, lamp circuits.

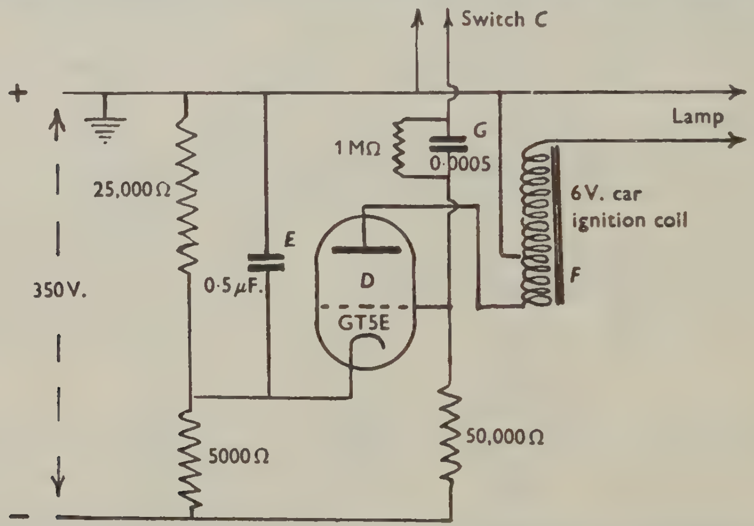


Text-fig. 2. Diagram of the rotary switches. The positions of the various brushes are indicated by arrows, and the switches are drawn in their correct relation for the direction of rotation as shown. (For details see text.)

The switching is carried out by three rotary switches, all mounted on the same shaft; these are shown separated in Text-fig. 2. The charging switch *A* is a Tufnol disk $2\frac{1}{2}$ in. diameter carrying brass inserts on opposite sides, each of these inserts occupying 90° of the circumference and being connected to a brass contact at the centre of the disk. Switch *A* is mounted on the end of the shaft. On the centre contact rests a spring brush which is connected to the power unit, while a second brush connects the flashing condenser with the peripheral segments. The condenser is, therefore, connected to the power unit for half the time cycle. The second switch *B*, which may be called the lamp switch, is mounted on the shaft close to switch *A*. It is a similar $2\frac{1}{2}$ in. Tufnol disk carrying two narrow brass inserts on opposite sides; these inserts are connected together by a copper strip as shown. Pressing against the periphery of the disk and 180° apart are two copper carbon brushes. One is connected to the flashing condenser, and the other leads to the lamp. The two switches *A* and *B* are adjusted relative to each other, so that the contact period of *B*, which occupies about $\frac{1}{20}$ of the cycle time, occurs in the middle of the off-period of *A*. The position of these switches in the circuit is shown in Text-figs. 5, 6. The third switch *C*, which times the flashing of the lamp, is similar to switch *B*, and carries two narrow contact strips on opposite sides. Two light spring brushes rest side by side on the periphery and the circuit between them is made by the contact strips. Each time this circuit is made a pulse is sent to the lamp from the trigger circuit to be described.

Switch *C* is adjusted to close when the contacts of switch *B* are under the brushes. The complete sequence of operations is as follows:

The cycle starts with a contact segment of *A* just touching the brush leading to the condenser; the power unit is thus connected to the condenser which begins to charge. This connexion is maintained for a further 90° of rotation by which time the charging is complete.



Text-fig. 3. Circuit of the trigger unit. The condenser *E* is charged from a simple power unit (not shown). When *C* is closed the grid of the thyratron *D* is momentarily raised to anode voltage through *G*. The valve becomes conducting and *E* is discharged very rapidly through the transformer *F*, giving a high-voltage pulse which is fed to the lamp.

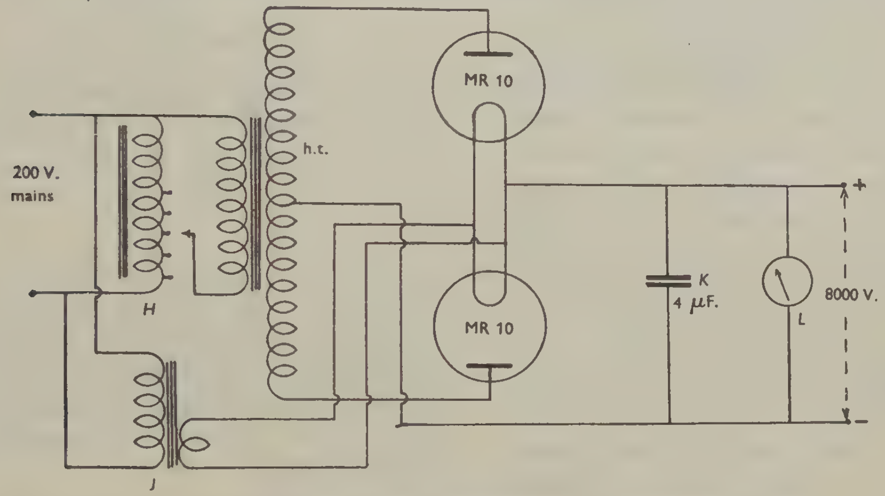
The switches continue to rotate through a further 45° when the circuit is made through switch *B* connecting the charged condenser to the lamp. While this circuit is made, the triggering pulse is released by the closure of switch *C*, and the lamp flashes. After a further 45° of rotation switch *A* is again made and the cycle restarts.

Thus two flashes occur during each revolution of the shaft.

The trigger unit is identical with that described by Edgerton (1932), and is shown in Text-fig. 3. The gas-filled relay *D* discharges the condenser *E* when switch *C* is closed. A pulse of current, therefore, passes through the primary of the transformer *F* and produces a high-voltage pulse (about 10,000 V.) in the secondary, which is connected to the lamp.

The power unit, Text-fig. 4, is very simple though rather large; it contains a 5 kVA. high-tension transformer fed from the tapped transformer *H* for voltage control. The centre-tapped secondary output can be varied up to about 18,000 V. by adjustment of *H*. This output is rectified by two diode rectifiers (Marconi M.R. 10), and smoothed by condenser *K*. The rectifier filaments are fed from transformer *J*. The resultant d.c. voltage is shown on the meter *L*. The complete unit is built into an angle iron framework, which is enclosed in sheet metal, and fitted with wheels. The latter are essential since the total weight is about 900 lb.

The camera was constructed from an old hand-cranked 35 mm. cine camera. The main modifications are as follows. The claws, all the intermittent mechanism, and the shutter were removed. A new film gate was fitted, and an additional sprocket was fitted in place of the intermittent mechanism, to pull the film smoothly and steadily through the gate. A focusing mounting was made to take a new lens (Zeiss Sonnar *f* 2·0). Finally, a coupling was fitted in place of the handle to connect the camera to the shaft carrying the switches. Since the camera was designed to take two pictures per revolution of its shaft, and the rotary switches produce two flashes per revolution, the synchronism between the lamp and camera is perfect.



Text-fig. 4. Circuit of main-power unit. For description see text.

The camera drive is carried out by a ¼ h.p. electric motor; this is larger than necessary, and therefore gives a very steady speed.

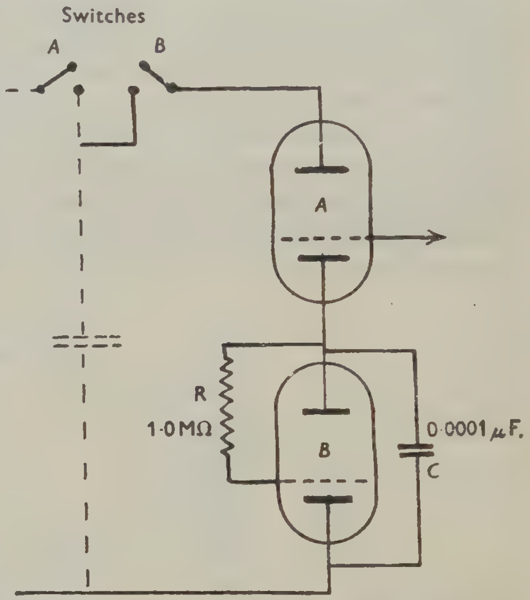
The drive is taken from the motor by a belt to a pulley on the switching shaft. The belt is normally slack, and slips while the shaft is held stationary by a powerful brake. An electromagnet is arranged to release the brake and tighten the belt simultaneously.

The lamp is mounted in a large motor head-lamp reflector on an adjustable stand, and is connected by flexible cables with the lamp switch and trigger unit. All the high-voltage cable is concentric radio-frequency transmission cable with about $\frac{5}{16}$ in. solid polythene insulation and has been tested to 30,000 V.

An alternative circuit using two lamps, to lessen the shadows on the screen behind the bird, was used for the photographs in this paper.

This circuit is shown in Text-fig. 5; the operation is as follows:

On lamp *A* being triggered, a discharge passes, charging condenser *C*, it also raises the voltage on the trigger electrode of lamp *B* via the resistance *R*. When this voltage is high enough the second lamp becomes ionized and the flashing condenser discharges through the two lamps in series.



Text-fig. 5. Circuit using two lamps. Lamp *A* is controlled by the trigger unit. Lamp *B* will flash whenever the voltage across it exceeds about 6000 V. Therefore, when *A* is triggered it starts to charge *C*, when the voltage across *B* is high enough, both lamps become conducting and the flashing condenser is discharged through both in series.

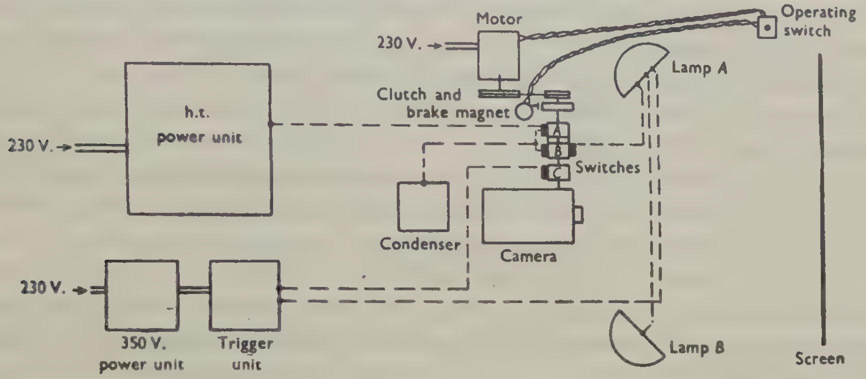
OPERATION

The sequence of operations is as follows. The trigger and power-unit filaments are switched on. The camera is focused and loaded with film. Then the trigger and power-unit high-voltage circuits are made, and the apparatus is ready for use. The motor and electromagnet are both operated by push switches. Actually these are combined mechanically so that the first movement of the push button against light spring pressure completes the motor circuit. The motor starts and reaches full speed, the bird is released, and the button pressed further down against a stronger spring. This further movement energizes the electromagnet, and the camera and switches start running.

Within $\frac{1}{10}$ sec. the camera has reached its running speed of 85–95 pictures per sec. On the completion of the shot the button is released; this applies the brake, slackens the belt, and switches off the motor. The camera is stopped by the brake in less than $\frac{1}{10}$ sec. The wastage of film at each end of a shot is not more than 8 in.

The complete layout with two lamps is shown in Text-fig. 6. The flashing condenser is variable in steps of 0·25 μF. The photographs illustrating this paper were taken using a condenser of 1·0 μF. charged to 8000 V. and required an output of about 4 kW. from the power unit. The shots varied in length from about 30 to 50 pictures.

Other features of the experimental set-up should be mentioned. First, some photographs were taken against a background ruled in squares (not shown) in order to obtain estimates of the extent and velocity of the wing movements. Secondly, the timing device seen in the corners of the photographs in Pls. 5 and 6 was very useful to measure the exact interval between pictures. It is a disk carrying an arrow and rotating in front of a larger disk which is subdivided into ten sectors as shown. The small disk is rotated by a synchronous motor at 25 rev./sec., so that it is possible to estimate the time interval to within $\frac{1}{500}$ sec.



Text-fig. 6. Diagram showing the layout and interconnexions of the separate units. The dotted lines represent concentric polythene cable. The switches are labelled as in Text-fig. 2. (Not to scale.)

FLIGHT ANALYSIS

The wing-beat cycle

It is necessary to consider the wing movements from two points of view. First, relative to the bird, in order to determine the movements of the skeleton and the time relations of the various muscular contractions which are involved in the movement. Secondly, relative to the air, in order to obtain some idea of the nature and magnitude of the aerodynamic forces to which the wing is subjected during the cycle.

Movement relative to the body

The wing-beat cycle may be seen in Pls. 5 and 6. Pl. 5, fig. 1 shows the condition slightly after the beginning of the down beat. The wing is fully extended and the alula is open, thus allowing a high angle of attack around the mid-point of the span (Graham, 1930). The chief muscle active at this phase is m. pectoralis major. The movement which is a simple rotation round the shoulder joint (Pl. 5, figs. 2, 3) continues until it reaches or slightly passes the horizontal (Pl. 5, fig. 4).

Next the tips are seen to swing forward (Pl. 5, fig. 5); during this forward swing the wing starts a complicated series of movements which produce the upstroke. By putting the wing of a pigeon into the positions shown in successive photographs it is possible to trace the skeletal movements which occur, and thus indicate the muscle or muscles which produce such movements. When the articulations of the wing are examined in detail it becomes evident that in the position of Pl. 5, fig. 4 the humerus has almost reached the limit of its downward and forward movement, and therefore that the subsequent forward swing must be associated with flexure within the wing. This flexure involves both the elbow and wrist (Pl. 5, figs. 5, 6). During this phase, also, the wrist is rotated so that the plane of the primary feather group becomes almost vertical and parallel to the long axis of the body, the muscles involved being m. biceps, flexor carpi ulnaris. By now the activity of m. pectoralis major has ceased, and this point may be taken as the end of the downstroke.

The upstroke is, as stated before, very complex, and so far it is by no means certain which muscles are most important and how flexible is the pattern of their activity. What happens to the wing as a whole is clear. There are at first three movements developing simultaneously: (1) an adduction of the humerus by m. pectoralis tertius, infra spinatus, and later latissmus dorsi; (2) a rotation of the humerus along its long axis so that the radius and ulna become almost vertical (Pl. 6, fig. 1), effected by m. pectoralis minor whose contraction has this result when the humerus is already adducted; (3) a further flexion and supination of the wrist (Pl. 6, fig. 2). When this folding and rotation of the wing is complete a very rapid backward flick ensues (Pl. 6, figs. 2–4). This movement is accomplished by an upward and forward rotation of the humerus around the shoulder joint, coupled with a rapid extension of the wing and pronation of the manus.

The speed of this phase is very high, as can be seen in the spacing of Pl. 6, figs. 2–4. It is clear from the bending of the feathers that the forces at this stage are large, and must be produced chiefly by the extension of the wing under the action of m. triceps and the more distal extensors; also that, since the flick produces a force tending to swing the humerus forward, the movement of the bone must be controlled by a retractor such as m. latissmus dorsi. At the end of the upstroke, where there is no evidence of appreciable aerodynamic forces, and the forward swing continues (Pl. 6, figs. 5, 6), it is probable that m. deltoideus completes the cycle and is followed by the next contraction of m. pectoralis major.

It is convenient for discussion to subdivide the cycle into a number of phases based on the changes of direction of the wing. The simplest scheme is as follows:

(1) *Downstroke*. Begins with the wings vertical, ends with them just below the horizontal and still fully extended (Pl. 5, figs. 1–4).

(2) *Forward swing*. Begins at the end of (1), includes the retraction and rotation of the manus, and ends with the wing tips facing forward and with their surfaces parallel (Pl. 5, figs. 4–6).

(3) *Changeover point*. This is the small time interval where the forward swing has finished and the next stage is about to begin (Pl. 5, fig. 6).

(4) *Backward flick*. This is the phase in which the flexed wing is moved backward and upward and at the same time extended. (Pl. 6, figs. 1–4).

(5) *Extension*. Here the wing, having completed (4), is extended fully prior to the repetition of (1) (Pl. 6, figs. 5, 6).

It is evident that a subdivision as above is quite arbitrary, since the wing in fact passes smoothly from one state to the next. Nevertheless, the output of useful work of the wing is not continuous, and can be related to the different phases described.

If the flight is photographed from in front as in Pls. 7 and 8, it is possible to clarify some of the more complex parts of the cycle.

In Pl. 7, fig. 1, about one-third of the downstroke has taken place. In Pl. 7, fig. 3, the forward swing is well developed, while in the next figure it is complete. This figure represents the changeover point and corresponds to Pl. 5, fig. 5. Pl. 7, fig. 5, shows the beginning of the backward flick. The flexion and rotation of the wing is clear in this and subsequent figures up to Pl. 8, fig. 1.

In the rest of Pl. 8 the downstroke can be followed through figs. 2–5, while in fig. 6 the forward swing has begun.

Text-figs. 7–12 show the approximate positions of the skeletal elements at various parts of the cycle. The drawings are taken from selected photographs, not necessarily of the same series, the top views being derived geometrically from the other two. The very limited movement of the humerus is evident, also the complicated flexion, rotation and extension during the flick in Text-figs. 10–12.

PATH RELATIVE TO THE AIR

The track and approximate angles of the primary feathers can be seen in Pls. 5–8 during the downstroke, while the separation of the feathers and their rotation during the upstroke is also visible.

There is here a complex sequence of movements which, under the conditions of slow flight, must give a resultant average force which is almost vertical but with a small forward component. In other words, there must be a force equal to the weight of the bird acting in opposition to gravity, and another horizontal force which provides the propulsion.

Since the forward velocity is low and fairly constant it is safe to assume that this component of the air reaction is small. It is clear from the deflexion of the feathers in the photographs, notably Pl. 7, figs. 1 and 6, that both the up- and downstrokes give lift. It is also evident in Pl. 6, fig. 3 and Pl. 7, fig. 6 that a forward component is present during the backward flick. On the other hand, only in Pl. 5, fig. 3 is there any suggestion of a forward force during the rest of the cycle, while in Pl. 7, fig. 3 the forward swing is seen to give a backward component.

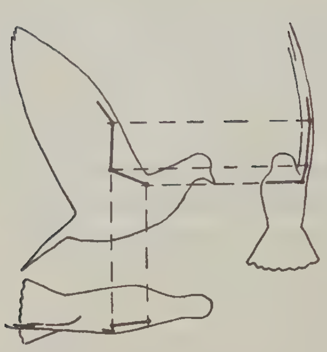
It would appear, therefore, that the force cycle is as follows:

During the downstroke there is an upward force with little or no forward inclination. As the wing passes into the forward swing the resultant force is tilted behind the vertical. Then as the upstroke develops the upward force which fell to zero at the changeover point is re-established and is accompanied by a fairly large forward force.

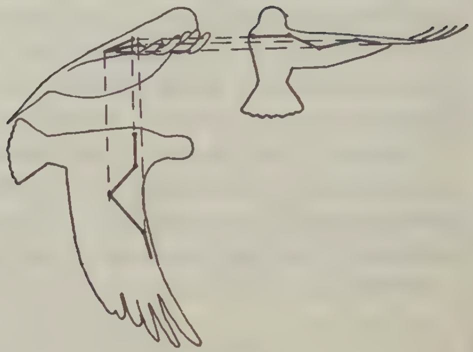
This forward force is maintained throughout the flick, while the lift probably dies away after the middle of this phase. Finally, both lift and propulsive components are absent during the extension.

DISCUSSION

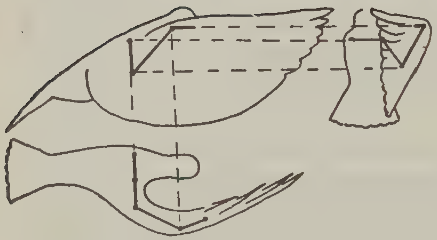
It must be emphasized that the type of flight which has been described is not the normal flight of the pigeon. It is the mode of flight used by the bird either just after take-off or just before landing, where in both cases the forward velocity is very low. It is certainly much more exhausting than fast flight, and cannot be maintained for more than a few seconds. In this type of flight it is probable that the secondary



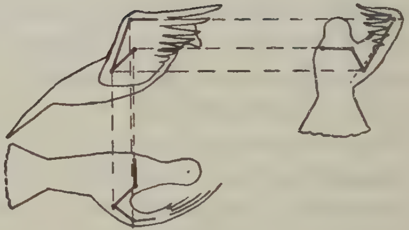
Text-fig. 7.



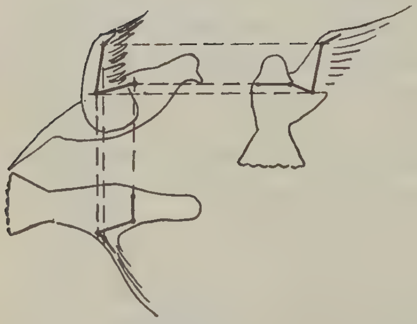
Text-fig. 8.



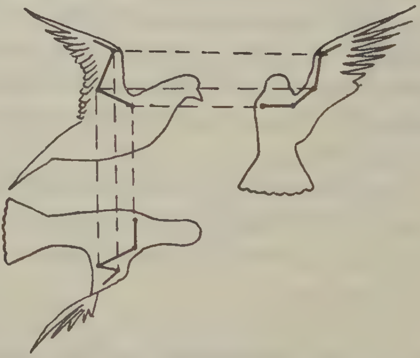
Text-fig. 9.



Text-fig. 10.



Text-fig. 11.



Text-fig. 12.

feathers are of little importance, since their velocity through the air is very low. This is confirmed by the work of Boel (1929), who showed that the slow flight was apparently not impaired when these feathers were removed, while flight was impossible when a part of the distal primaries was cut off. It is suggested, therefore, that the pigeon in slow flight uses only its primary feathers, and that it obtains lift and most or all of the propulsion from the upstroke, but only lift from the downstroke. Since the bird tires very rapidly the flight must either be very inefficient, or else must throw an undue amount of work on to muscles which are not highly stressed in fast flight.

Until adequate photographs of fast flight are available for study it is only possible to guess at the differences between the two types.

The points of difference which might be expected are (1) the disappearance either wholly or in part of the backward flick, and (2) the transfer of the propulsive function to the downstroke. If this proves to be the case, the work of all the muscles mentioned, except m. pectoralis major and perhaps m. deltoideus, is much reduced, and maintained flight becomes possible.

The path of the wing has been described by many authors and there is general agreement on the course of the down beat, but on the movements and function of the upstroke there are a large number of different opinions. These divergent views arise in two ways: first, the speed is high, making the movement difficult to photograph and impossible to see; and secondly, due to the occurrence of at least two types of upstroke. If consideration is limited to those accounts which describe a movement accompanied by a separation of the primary feathers, the conclusions reached by Headley (1912), Marey (1890), Horton-Smith (1938), and Guidi (1938) appear to be that the primary feathers separate on the upstroke in order to reduce the air resistance. Only Lorenz (1933) seems to postulate any upward force being developed by the wing tip during this part of the cycle. I have some evidence from wind-tunnel tests, which will be dealt with in a separate paper, which suggests that the group of feathers on the hand is behaving as a multi-slotted wing as described by Handley-Page (1921). If this is correct, then the wing under such conditions would have a high value of lift and also a large drag, but this drag would tend to propel the bird forward, since the wing is moving in the opposite direction to the body. This drag may, in fact, be the only source of propulsive component in slow flight.

Finally, there is a point of some functional importance in connexion with the

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Explanation of Text-figs. 7–12

Text-figs. 7–12. Diagrams of the skeletal movements during the flight cycle. Each figure shows the bird viewed from three directions at right angles.

Text-figs. 7–9. Show downstroke with wing fully extended, followed by forward swing. Note that the forward movement is achieved by flexure of elbow and pronation of manus.

Text-fig. 10. Beginning of flick. Shows further flexion of the wing and the backward and upward rotation of the humerus.

Text-fig. 11. Further development of propulsive flick. Continued rotation of the humerus and supination of hand.

Text-fig. 12. Completion of upstroke. Extension of the wing is nearly complete and the manus is returning to the position of Text-fig. 7.

insertion of m. pectoralis major. It has been noted by many workers, for example Headley (1912), that the insertion is on the forward face of the humerus, and that 'Its pull, therefore, tends to lower the front part of the wing relative to the hind part by rotating the bone'.

From the examination of the movement this view appears to be incorrect.

The humerus is never at right angles to the body; it is a fact that during the downstroke, when the wing is fully extended, it makes an angle of not more than 50–60° with the long axis of the body. As a result the pull of the muscle, while clearly tending to rotate the bone, does not necessarily tend to rotate the wing as a whole. In other words, the fact that the humerus has a backward inclination makes its rotation a normal occurrence during the lowering of the wing.

SUMMARY

1. A new high-speed photographic method is described in detail.

2. Illustrations show the results obtained by the application of the technique to the study of bird flight.

3. A detailed account is given of the movements involved in the flapping cycle, with an attempt to indicate the principal muscles active at different parts of the cycle.

4. The basic conditions and limitations of the slow flight of the pigeon are discussed, and the expected differences between it and fast flight are indicated.

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EXPLANATION OF PLATES

Plates 5 and 6. Pigeon, side view. Ninety-one pictures per sec.

Plate 5

Fig. 1. Wing in early part of the down beat. The alula is opened, and there is evidence of a forward force on the first two primary feathers.

Fig. 2. Wing horizontal. Forward force not shown.

Fig. 3. Further downward movement. Note the twist of the wing and the beginning of the forward swing. There is probably a slight forward component of force.

Fig. 4. Downward movement complete. Forward movement developing. The bastard wing is still open and the tip is still bent upward, showing that the wing is still active.

Fig. 5. Commencement of the flexure which marks the beginning of the upstroke. Note that the tip feathers are still bent due to pressure on the lower surface.

Fig. 6. Upstroke developing. Flexure of elbow and wrist increasing. Note that the feathers are uncurved, showing that no external work is being done.

Plate 6

Fig. 1. Upstroke further developed. Flexion has reached its maximum, and the flick is commencing. Note the rotation and bending of the feathers, showing that the air-flow has reversed and an upward force is present.

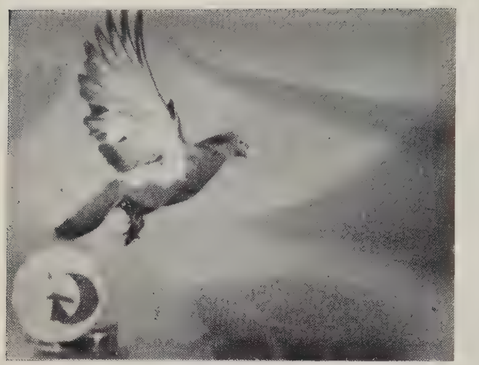


Fig. 1

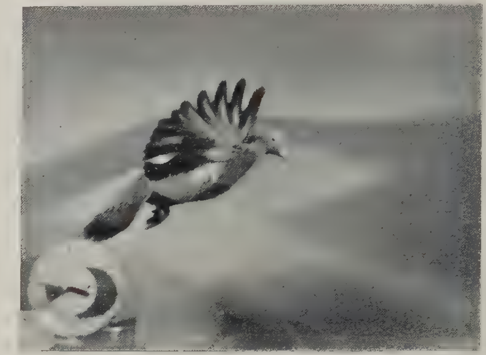


Fig. 2

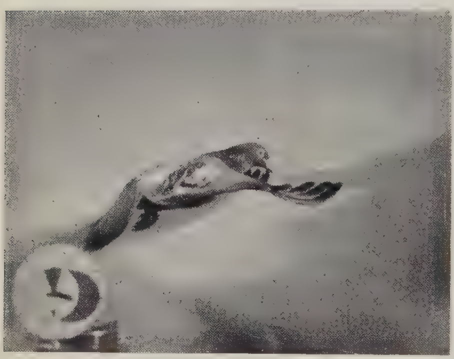


Fig. 3

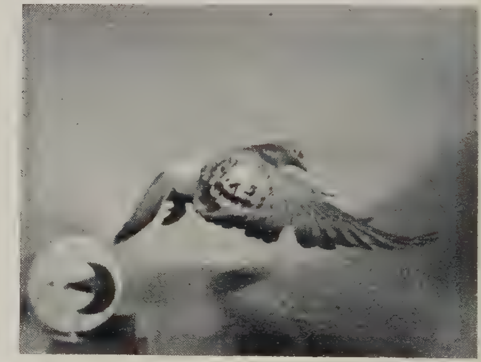


Fig. 4

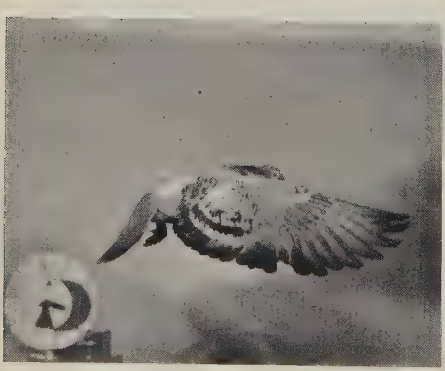


Fig. 5

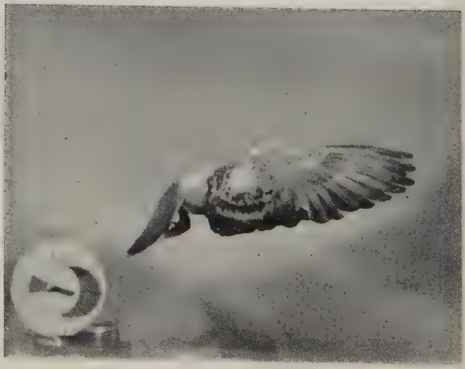


Fig. 6

BROWN—THE FLIGHT OF BIRDS

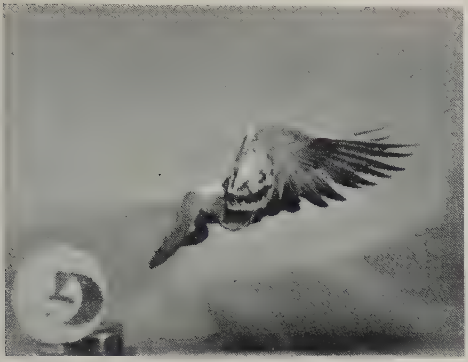


Fig. 1

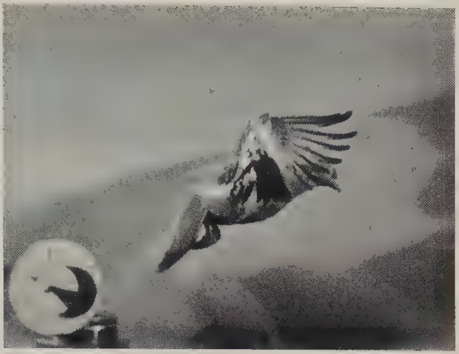


Fig. 2

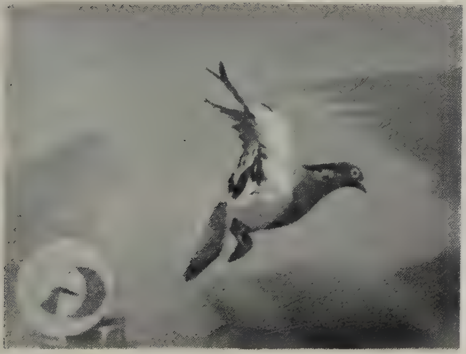


Fig. 3

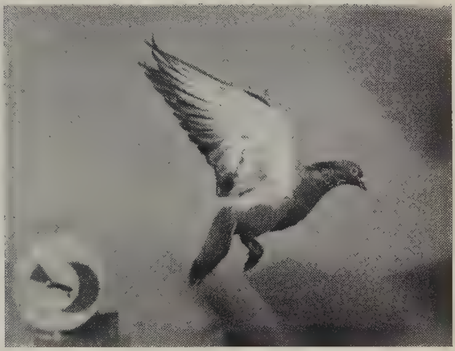
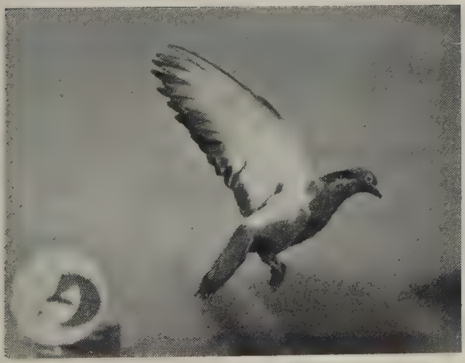


Fig. 4



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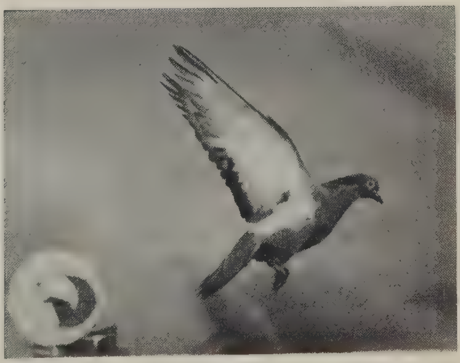


Fig. 6

BROWN—THE FLIGHT OF BIRDS

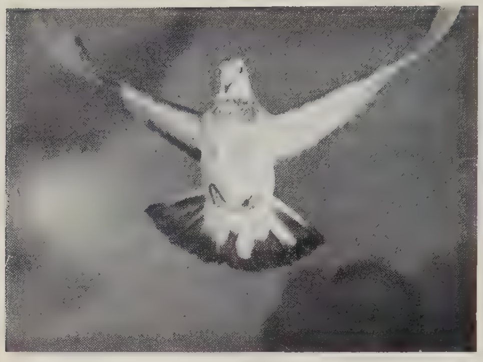


Fig. 1

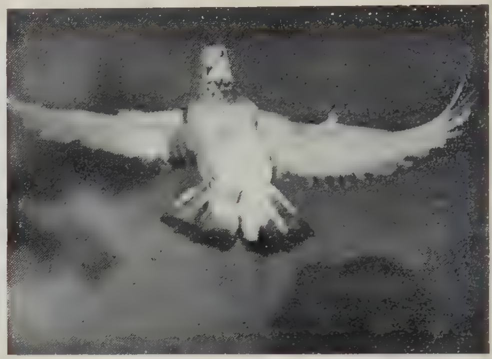


Fig. 2

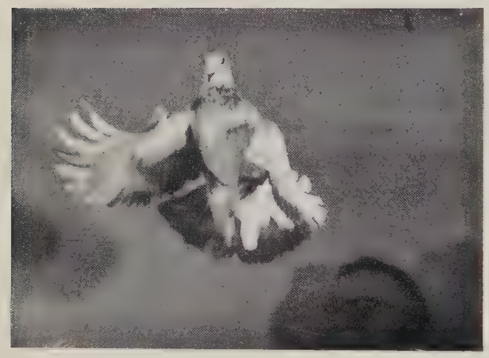
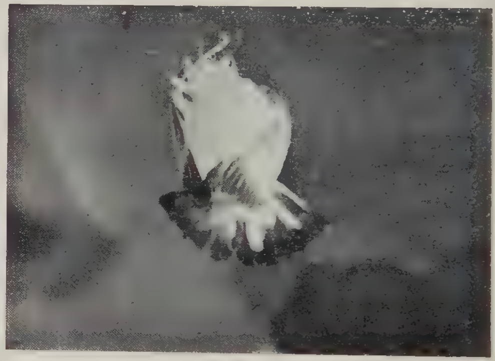


Fig. 3



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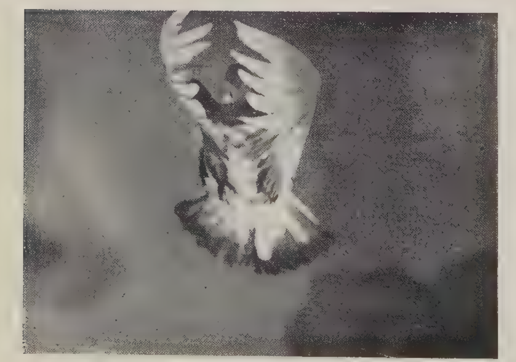

Fig. 5

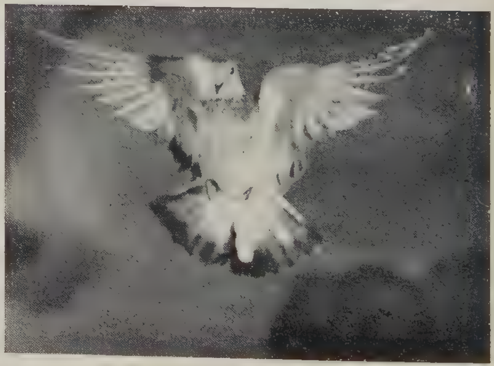

Fig. 6

BROWN—THE FLIGHT OF BIRDS

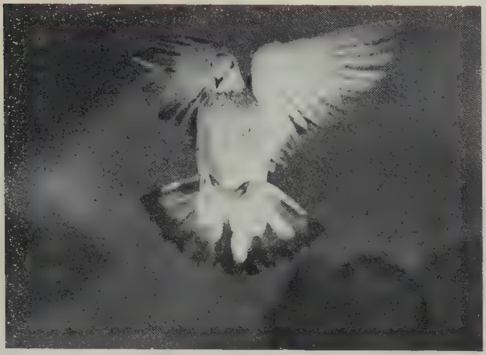

Fig. 1

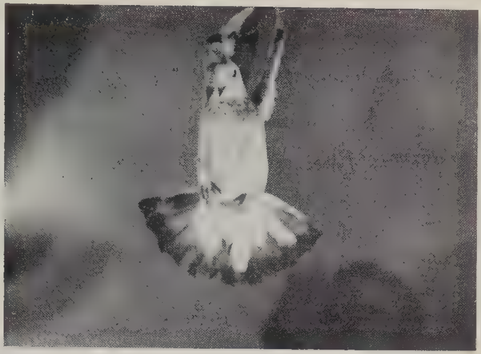

Fig. 2

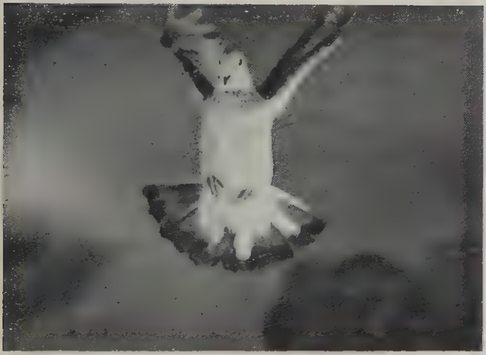

Fig. 3

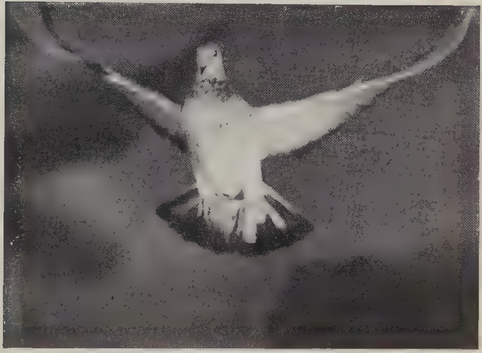

Fig. 4

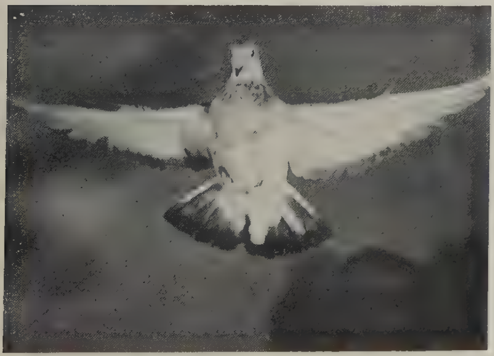

Fig. 5

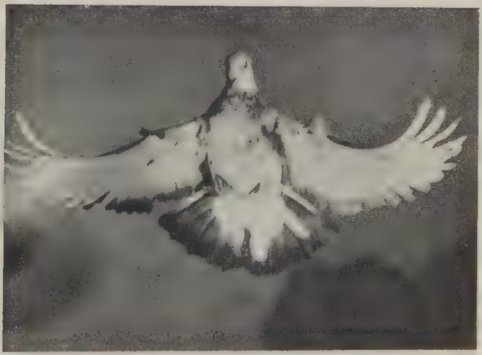

Fig. 6

BROWN—THE FLIGHT OF BIRDS

Fig. 2. Flick phase of upstroke. Note the rise of the wing as a whole and the upward force on the feathers.
Fig. 3. Upstroke nearly complete. Wing is extending while an upward force is still evident.
Fig. 4. Completion of upstroke. Wing not yet fully extended. Slight bending of tip feathers, therefore movement is still taking place.
Fig. 5. Passive phase with wing extending.
Fig. 6. Beginning of downstroke. Alula is just starting to open.

PLATES 7 and 8. Pigeon, front view. Eighty-five pictures per sec.
Compare with equivalent photographs in side view.

PLATE 7

Fig. 1. Approximately equivalent to Pl. 5, fig. 2.
Fig. 2. Approximately equivalent to Pl. 5, fig. 3.
Fig. 3. Approximately equivalent to Pl. 5, fig. 5.
Fig. 4. Approximately equivalent to Pl. 5, fig. 6.
Fig. 5. Approximately equivalent to Pl. 5, fig. 6 and Pl. 6, fig. 1.
Fig. 6. Approximately equivalent to Pl. 6, fig. 2.

PLATE 8

Fig. 1. Approximately equivalent to Pl. 6, fig. 3.
Fig. 2. Approximately equivalent to Pl. 6, fig. 5.
Fig. 3. Approximately equivalent to Pl. 5, fig. 1.
Fig. 4 as Pl. 7, fig. 1.
Fig. 5 as Pl. 7, fig. 2.
Fig. 6 between Pl. 7, figs. 2 and 3.

THE REGENERATION OF LEGS IN *RHODNIUS PROLIXUS* (HEMIPTERA)

By MARTIN LÜSCHER

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(*Received 22 June* 1948)

(With Plate 9 and Seven Text-figures)

It is well known that many insects, and especially many Orthoptera, are capable of regenerating autotomized and amputated legs (Godelmann, 1901; Bordage, 1905; Megusar, 1910; Friedrich, 1930). Very little is known, however, about those factors governing regeneration, those initiating the excessive growth in regeneration, and those suppressing growth when the regenerated leg has reached a normal size. In order to investigate these factors and their mode of action, it is very convenient to have an insect in which the hormonal control of moulting and metamorphosis is well known. Such an insect is *Rhodnius* (Wigglesworth, 1936, 1940*a*, *b*), and this insect is also able to regenerate the distal parts of its legs. As regeneration in *Rhodnius* has not previously been described, it has been necessary to study the normal process of repair in relation to time, temperature and amputation level in order to provide a basis for further work on the factors mentioned above. Normal regeneration will be described in this paper. Strangely enough the temperature and time relations have never been investigated in insect regeneration, although much physiological work has been carried out on the subject.

METHODS

Each insect was kept separately in a glass tube and fed separately, so that the complete individual history was known. Apart from the dates of moulting and feeding, the records included the measurements of all parts of the amputated and the control leg in each instar. The exuviae, on which all external details of the regenerating leg can be seen, were prepared together with the regenerated leg of the adult so that every stage in regeneration could be seen in a single preparation (Pl. 9). All experiments were carried out on the second pair of legs. No significant differences could be detected between the regeneration potencies of the different legs. As a rule the insects were kept at a temperature of 25° C. and at a relative humidity of 50–60%.

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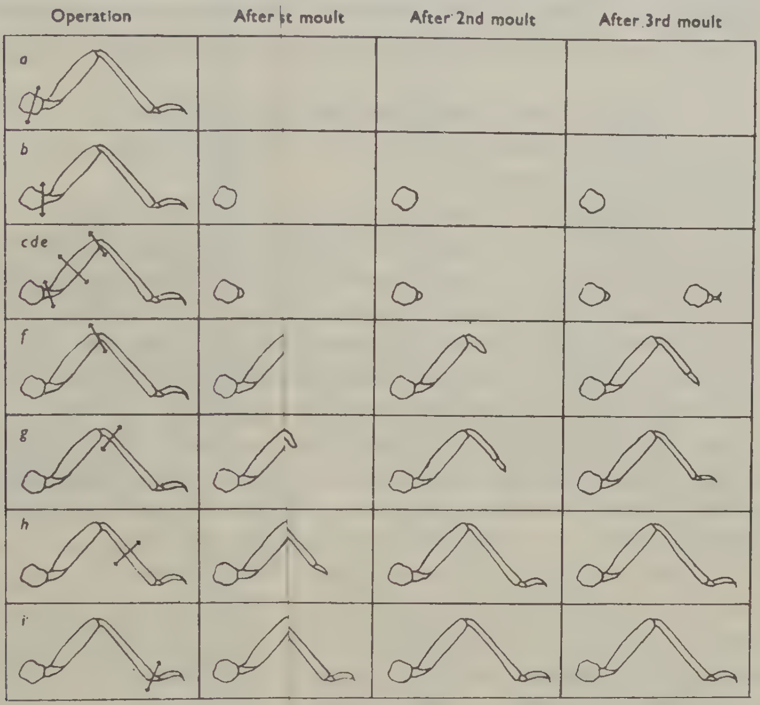
* I should like to thank Dr V. B. Wigglesworth for providing laboratory facilities in his department and for many helpful suggestions. The work was carried out with the help of a grant from the 'Schweizer Stiftung für biologisch-medizinische Stipendien'.

The regeneration potencies of the leg

The possibilities of regeneration in a leg of *Rhodnius* are summarized in Text-fig. 1. The operation, which is carried out at the time of feeding, is shown in the first column. The other three columns show the regeneration resulting after the next three moults. The figure is valid for any nymphal stage, no striking differences in the regeneration potencies of the different nymphal stages being detectable.



Text-fig. 1. The possibilities of regeneration in a leg of *Rhodnius prolixus.*

If a leg is amputated through the coxa (*a*), the remaining part of the coxa is eliminated and no regeneration occurs. After amputation between coxa and trochanter (*b*), the coxa remains intact but no regeneration occurs. If the leg is cut off through the trochanter (*c*), a part of the latter persists. Amputation through the femur (*d*) causes the elimination of the remaining part of the femur and of a part of the trochanter; as a rule no regeneration takes place after this, but occasionally a small rudiment with end-claws appears at a late stage. If the leg is cut off between femur and tibia (*e* and *f*), there are two possibilities: either the whole femur and a part of the trochanter are eliminated (*e*), or the femur remains intact after the next moult (*f*). In the latter case regeneration begins to be visible after the second moult when a small rudimentary tibia is formed. A more complete leg is regenerated after the third moult. After an amputation through the tibia (*g*, *h*, *i*) regeneration is more rapid and begins after the next moult. The figure shows that the capacity for regeneration increases gradually the more distally the amputation level lies. If the proximal part (*g*) of the tibia is amputated no tarsus is formed after the next moult, while a tarsus of one segment is formed after amputation in the middle of the tibia (*h*) and a tarsus with two segments after amputation in the distal part of the tibia.

We may conclude from this account that *Rhodnius*, unlike Phasmids and Mantids, is not capable of complete regeneration but only of partial regeneration of its legs. The regeneration potencies appear only at the level of the femur-tibia joint and thence increase gradually in a distal direction. This does not mean that if the tip of the leg is cut off there will be a quantitative maximum of regeneration, but differentiation of the regenerated part will be more advanced the more distal the operation.

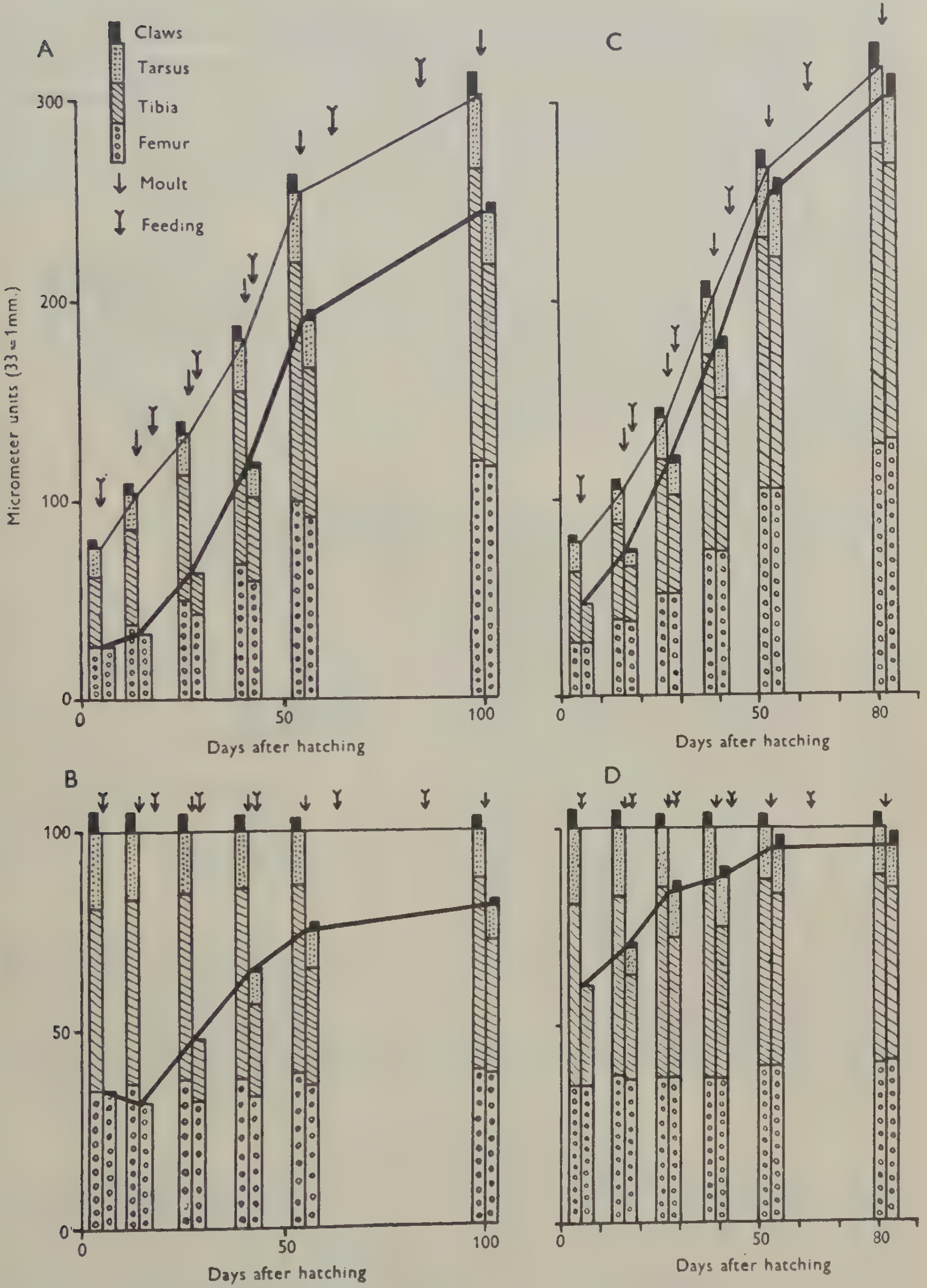
Description of two typical cases of regeneration

In order to see how regeneration proceeds after it has been initiated by amputation we may study two typical cases.

In Pl. 9A the regeneration of case 59*r* is shown from the first instar to the adult. This is a photograph of the exuviae of each nymphal instar and of the leg of the adult. The leg was amputated between femur and tibia at the time of feeding. After the first moult nothing has happened except that the wound is completely closed and the cuticle of the whole femur is much thinner, softer and less darkly pigmented. A short tibia has been formed after the second moult, and after the third moult the regenerated part consists of a tibia and a tarsus of one segment with claws. It is only after the fourth moult that the regenerated leg is complete with a tarsus of two segments. But in the following adult stage the tarsus of the regenerated leg still consists of two segments, while that of the control leg has acquired its third segment. Regeneration is therefore incomplete; the regenerated leg has not reached the length of the control leg and one tarsal segment is missing. It is also noteworthy that after five moults the cuticle of the whole leg, i.e. even that of the femur which has not been regenerated, is much softer and clearer than that of the control leg.

The measurements of the regenerating leg and of the control leg in the same case (59*r*) are shown in Text-fig. 2A. The times of moulting and feeding are also indicated in this graph, in which the absolute lengths of the different parts of the leg are given in micrometer units. Regeneration and normal growth are combined in this figure, and I have therefore drawn in Text-fig. 2B the length of the regenerating leg as a ratio of the length of the control leg. The changes due to normal growth have therefore been eliminated in this figure. The figure shows that after the first moult not only is there no regeneration but the femur decreases relatively in length, and only after the second moult is the excessive growth of regeneration evident. This continues until the fifth nymphal instar is reached, when this hypertrophy comes to an end, although the regenerated leg is still far shorter than the control. It seems to be a general rule that regeneration ceases after the fourth moult if the greater part of the tibia is amputated. If only a short part is amputated, regeneration is terminated earlier, but the regenerated leg will never be quite as long as the control.

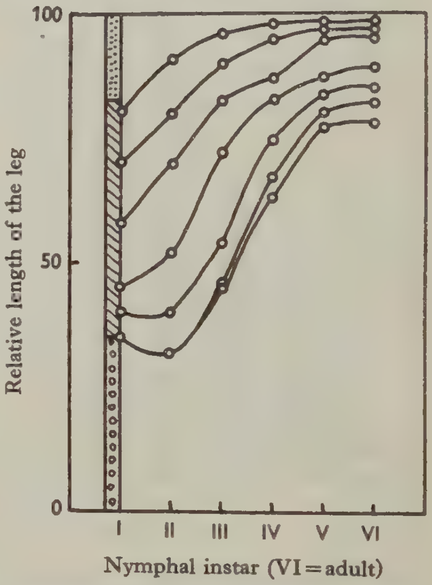
A further case (58*g*) in which regeneration was much more rapid is shown in Pl. 9B and in Text-fig. 2C, D. In the first nymphal instar the tibia was amputated in the middle at the time of feeding. After the next moult a tarsus with claws was



Text-fig. 2. The normal regeneration after amputation at the femur-tibia joint (A, B) or through the tibia (C, D). The figure shows the real growth of the normal and the regenerating leg (A, C) and the relative growth by regeneration (B, D).

already present. After the second moult the tarsus consisted of two segments, and in the adult the tarsus of the regenerated leg, as in the control leg, consisted of three segments. Here, therefore, we have a case of complete regeneration although the normal length is not reached (Text-fig. 2D). In this case regeneration also comes to an end after the fourth moult, but the excessive growth begins at once while in the first case it appears only at the second moult.

Text-fig. 3 shows a comparison of some similar cases in which the leg has been amputated at different levels of the tibia in the first nymphal instar. The figure shows that the maximum rate of regeneration occurs after the first or the second moult when the tibia has reached a length of 10–15% of the whole length of the leg. An increase of 20% and more is then possible between successive moults.



Text-fig. 3. Regenerative growth in cases where amputation was performed at different levels of the tibia.

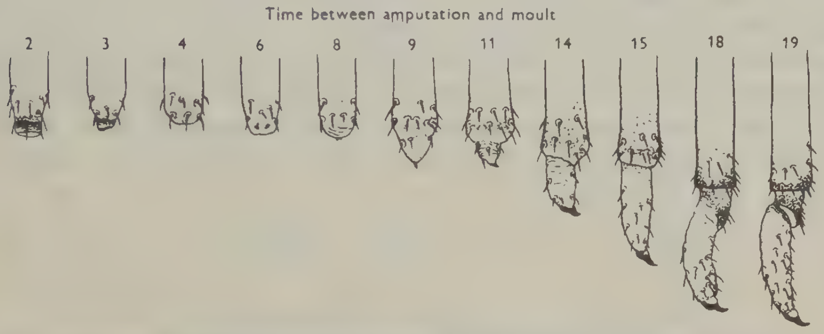
Regeneration in the different nymphal instars

It has been pointed out already that there are no striking differences in the regeneration potencies of the various nymphal stages. This might be illustrated by some experiments carried out on first, third and fifth nymphal instars. The tibia was amputated near the middle at the time of feeding. The figures in Table 1 give the relative increase in length of the regenerating legs after the first post-amputation moult. There is great variability between the individuals at the same nymphal instar. The average relative increase is greater in the late than in the early instars, but since in the former moulting occurs only after a longer period, we find that the relative increase per day is actually less, although of course more tissue is regenerated during the whole period between successive moults. The difference in regenerative capacity is significant, but there is no significant difference in the degree of differentiation of the regenerated leg after the moult which follows the amputation.

The time relations in regeneration

As was first pointed out by Schaxel & Adensamer (1923), the differentiation of the regenerated leg is dependent on the time which elapses between the amputation and the next moult. In spite of its obvious significance, however, the relationship has never been investigated experimentally. For this purpose *Rhodnius* is an ideal insect, since the time of moulting is dependent on the time of feeding and can therefore be controlled (Buxton, 1930).

The legs of a number of third instar nymphs were amputated through the middle of the tibia at different times before and after feeding. All stages of regeneration from an unhealed wound to a well-differentiated tarsus of two segments resulted. This is shown in Text-fig. 4. If the moult occurs 2 or 3 days after the amputation, the wound does not heal properly, though the edges of the new cuticle are gradually drawn together. Some haemolymph will flow from the wound and close it by coagulation. After a moult occurring 4 days after the amputation, the wound is perfectly healed and a continuous cuticle is formed. With an interval of 6 days very small claws begin to appear at the end of the tibia, while a slight separation between tibia and tarsus can be detected with an interval of 9 days. With 11 days a diminutive tarsus is clearly separated from the tibia. With 15 days the tarsus is much longer but still consists of one segment only. A tarsus of two segments is formed with an interval of 18 days.

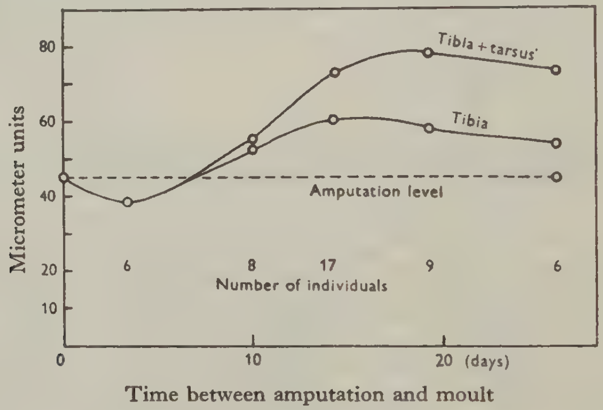


Text-fig. 4. All stages of regeneration after amputation in the middle of the tibia, obtained by varying the time interval between amputation and feeding.

Table 1. *Comparison of the regeneration in different nymphal instars*

Nymph of ...	First instar		Third instar		Fifth instar	
	Moult after (days)	Increase (%)	Moult after (days)	Increase (%)	Moult after (days)	Increase (%)
Figures for single individuals	9	8·1	14	5·1	21	16·6
	9	10·0	17	19·6	22	15·5
	9	9·4	15	13·0	23	15·0
	11	9·3	18	7·9	22	14·1
	9	8·2	15	15·3	22	14·7
	8	8·6	14	9·6	22	18·7
	11	15·3	16	15·7	24	13·0
	10	7·4	17	12·7	22	16·2
	9	8·7	14	12·1	23	11·3
	10	7·5	15	10·4	26	16·6
	10	7·9	18	13·8	25	15·8
	8	5·3	—	—	26	14·8
	—	—	—	—	25	15·4
Average	9·4	8·8	15·7	12·35	23·4	15·2
Average increase per day (%)	0·94		0·79		0·65	

The increase in length of the regenerated leg after different time intervals is shown graphically in Text-fig. 5. The maximum rate of regeneration is reached when moulting occurs 20 days after the operation, i.e. if the operation has been carried out about 5 days before feeding. If the amputation is performed earlier, regeneration is less efficient. It is therefore very probable that 5 days after the amputation the activation by the initiating factor is in full operation, so that a new activation by feeding will fall at just the right time to produce excessive growth. The height of cell activity during wound healing in *Rhodnius* also occurs 5 days after the injury (Wigglesworth, 1937). The same phenomenon has also been demonstrated in tadpoles of the anuran *Xenopus laevis* which have been kept without food for a certain time. Five days after the tails of these tadpoles had been amputated, mitotic activity among the cells of the stump reach a peak (Lüscher, 1946).



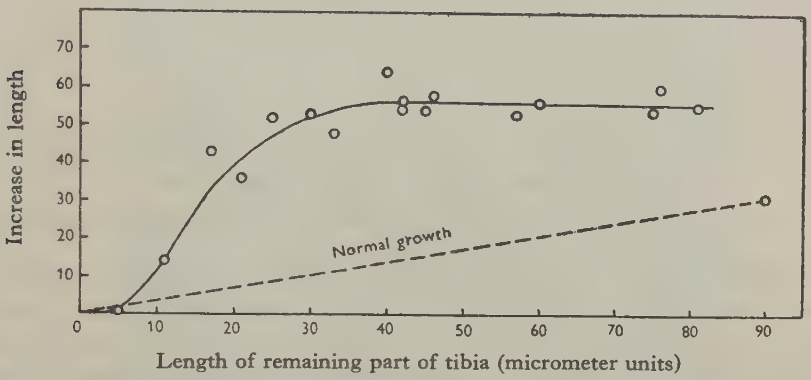
Text-fig. 5. The increase in length of the regenerating leg in relation to the time interval between amputation and moult.

The temperature relations in regeneration

Since the time of moulting is dependent upon the temperature, it is obvious that this factor also influences regeneration. It was expected that regeneration would be accelerated by high temperatures to the same extent as the moulting processes. But experiment has shown that the degree of acceleration is much more rapid in the case of regenerative growth. Three lots of third instar nymphs were fed and their legs amputated through the middle of the tibia. They were then exposed to temperatures of 17, 25 and 30° C. The results are shown in Table 2. The figures show clearly that a high temperature is very favourable for regeneration while a low temperature of 17° C. is unfavourable, even though the time which is available for regeneration is relatively long. The regeneration processes are therefore more dependent on temperature than the moulting processes.

The amputation level and regeneration

We have already mentioned the fact that the regeneration potencies increase progressively as the amputation level passes towards the tip of the leg. This obviously can only be true for the differentiation of the regenerated part and not for the quantitative regenerative growth. In order to investigate the relation of the amputation level to the regenerative growth, the legs of a number of fourth instar nymphs were amputated at different levels of the tibia at the time of feeding. Text-fig. 6 shows the results of this experiment. It gives the total increase in length of the tibia and the tarsus, i.e. the regenerating part which is formed in the remaining portion of the tibia, and represents the sum of regenerative and normal growth. A maximum is reached when the stump of the tibia has a length of 40 micrometer units. From this point the curve continues horizontally. From this we may conclude that the whole growth, normal and regenerative, does not require more than a part of the tibia which is 40 units long, and that only the distal part (40 units long) of the tibial stump takes part in regeneration.



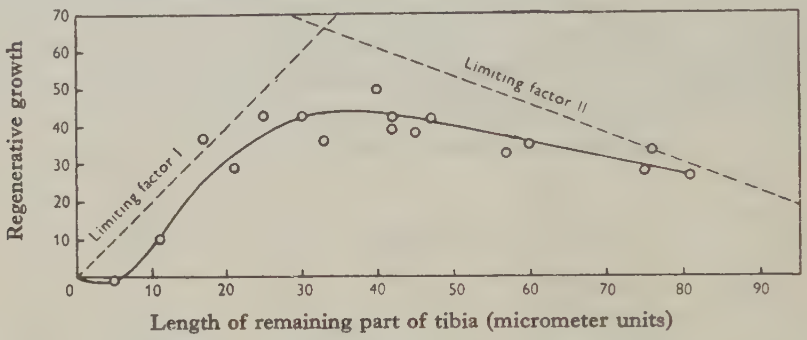
Text-fig. 6. The regeneration after amputation at different levels of the tibia; total growth.

Table 2. *The influence of different temperatures on regeneration*

Temp. (° C.)	17	25	30
No. of insects	8	17	9
Av. moult after (days)	42	14·2	12·3
Av. increase in length* of tibia (micrometer units)	3·5	15	17
Av. length of tarsus (micrometer units)	9·5	13	15·5
Av. total increase* (micrometer units)	14	28	32·5
Av. no. of tarsal segments	1	1	2

* The increase in this case is measured from the middle of the tibia after the moult. It therefore consists of regenerative growth only.

If we deduct from this growth increment the values for normal growth (which fall on a straight line), we obtain a curve with a definite maximum of regenerative growth when the length of the remaining part of the tibia is about 35 micrometer units (Text-fig. 7). We may suppose that there are two factors limiting regenerative growth: (i) the volume of the tibial stump and (ii) the length of the missing part, i.e. only a certain proportion of the growth required for replacement of the missing part will be accomplished. Obviously there must be a zone where both limiting factors (they may be thought of as gradients) are equal, and in this zone there will be a maximum of regenerative growth. The limiting factors may be drawn as straight lines as has been done in Text-fig. 7. According to this figure the first limiting factor, the space factor, would be about 200%, i.e. a regenerative increase of not more than 200% is possible in the remaining part of the tibia. The second limiting factor would be about 76%, i.e. not more than 76% of the required regenerative growth for the re-establishment of the normal length can be accomplished in the time available.



Text-fig. 7. The regeneration after amputation at different levels of the tibia; regenerative growth only.

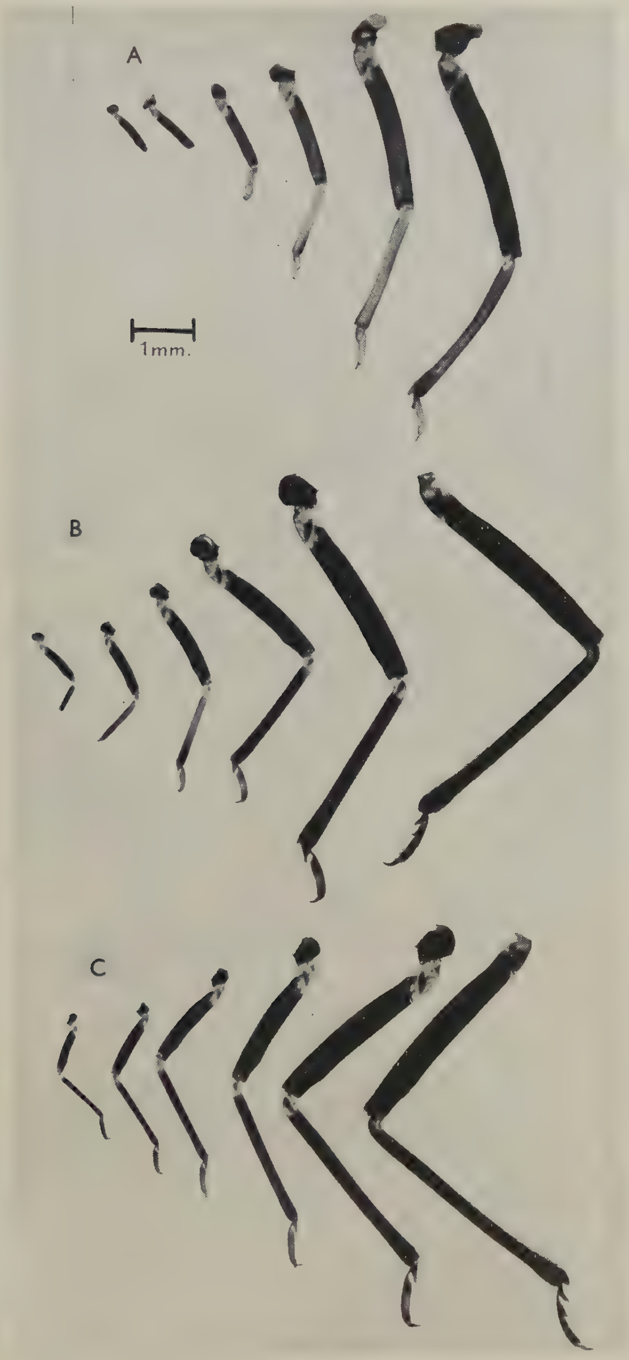
This conception of two limiting factors is not in accordance with our first conclusion, namely, that only the distal part of the remaining portion of the tibia takes part in growth and regeneration. At the present moment it is not possible to decide which conception is correct. Nevertheless, as there is no evidence for normal growth taking place only in a part of the tibia, the conception of the two limiting factors may prove a useful working hypothesis.

DISCUSSION

As *Rhodnius* has been shown to be capable of a partial regeneration of its legs, this function has now been demonstrated in an hemipteran. Very probably partial regeneration is also possible in many other insects. It also occurs, for example, in the milkweed bug *Oncopeltus fasciatus*, though the extent of regeneration is relatively slight (unpublished).

Partial regeneration becomes more complete the more distally the amputation level lies. The leg of *Rhodnius* may be regarded as possessing a gradient of regenerative potencies which is highest in the end of the tarsus. In this insect, therefore, the mechanism seems to be quite different from that in Phasmids where, according to Friedrich (1930), regeneration is much quicker if the leg is autotomized in the trochanter than if it is amputated at a more distal level. In Phasmids, however, regeneration and the correlated mechanism of autotomy are highly specialized acquisitions. In *Rhodnius* we may be dealing with a very primitive form of regeneration.

Regeneration in *Rhodnius* is dependent on the time interval between the amputation and the next moult, and almost any state of differentiation of the regenerated



LÜSCHER—THE REGENERATION OF LEGS IN *RHODNIUS PROLIXUS* (HEMIPTERA)

part can be produced by varying the time relations. A maximum of regeneration has been found when the insects were fed 5 days after the operation, probably when the initiating factor has reached its highest activity. Regeneration is also dependent on temperature. It can be almost entirely suppressed by low temperature while it is considerable at high temperatures.

The differentiation of the regenerated leg is also dependent on the amputation level. After amputation of part of the tibia the regenerative growth seems to depend on the volume of the tibia that remains and on the length of the missing part, only a certain proportion of which can be regenerated.

For future work on the factors governing regeneration we may conclude that it will be very important to keep the insects at a constant temperature, and to amputate the leg always at the same level and at the same time in relation to the time of feeding. But even so there may be considerable individual differences, so that it will be necessary to perform an extensive series of experiments and to analyse the results statistically.

SUMMARY

1. Partial regeneration of the legs is possible in *Rhodnius prolixus*.

2. The regeneration potencies appear at the level of the femur-tibia joint and increase gradually in a distal direction.

3. Regeneration usually ceases after the fourth post-amputation moult.

4. No striking differences in regenerative capacity can be detected either in the different legs on one nymphal instar or in the legs of different nymphal instars. There is, however, considerable individual variation.

5. Regeneration is dependent on the time which elapses between the amputation and the next moult.

6. The regeneration processes have a higher temperature coefficient than the moulting processes.

7. A conception of two limiting factors is briefly discussed.

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EXPLANATION OF PLATE 9

Two cases of regeneration in *Rhodnius prolixus* after amputation of the leg at the femur-tibia joint (A) and through the tibia (B). C, the normal growth of a control leg. Photographs of the exuviae and the leg of the imago.

THE PHYSIOLOGY OF SEA-URCHIN SPERMATOZOA

LACK OF MOVEMENT IN SEMEN

By LORD ROTHSCHILD

From the Department of Zoology, University of Cambridge

(*Received* 29 *June* 1948)

(With Two Text-figures)

INTRODUCTION

The eggs and spermatozoa of many organisms contain and secrete substances with fairly well-defined physiological properties. A knowledge of the chemical and physiological characteristics of these substances may lead to a more detailed understanding of the fertilization reaction. In addition, they are of interest from other points of view. On the one hand, they may contribute to our knowledge of the mechanism of vibratile movement; on the other, they may shed some light on immunochemistry, as the work of Tyler (1948) has shown.

The secretions of eggs, particularly in the case of marine worms and sea-urchins, were originally called Fertilizin. Eggs were also said to contain Antifertilizin, and so were spermatozoa. At other times, the words Agglutinin, Iso-agglutinin, and Hetero-agglutinin have been used to describe these secretions. Although on historical grounds it might be held that the names Fertilizin and Antifertilizin have a prior claim, they are somewhat cumbersome, if not confusing. Fertilizin may include separate substances with different properties, while Antifertilizin is obtained from both eggs and spermatozoa. Hartmann & Schartau (1939) have suggested that the secretions of eggs and spermatozoa should be called Gynogamones (G.) and Androgamones (A.) respectively. These names might be thought to prejudge several issues, for it is not certain that all the gamete secretions are hormones in the usual sense (gamone = gamete hormone); nor is it yet clear that all the secretions have a gamete significance. Nevertheless, the nomenclature is convenient and often used in Sweden, Germany and Italy. In Table 1,* which summarizes existing gamete secretion data, the new nomenclature is adopted, but the old names are included for comparison and identification.

Table 1 contains two new and debatable features. First, Hetero-agglutinin is included as a separate substance from Iso-agglutinin. This is based on Just's observation (1930) that Hetero-agglutinin can be removed from *Arbacia* egg water by *Nereis* spermatozoa, though the full amount of Iso-agglutinin is left in the egg water. Secondly, a new substance, Gynandrogamone I (Antifertilizin), has been added to the list. The reasons for the name will be clear from Table 1. The properties of this substance are strikingly similar to those of A. II, but according to Tyler (1939), GA. I can be separated from A. II. In our present state of knowledge, it therefore seems advisable to consider GA. I and Hetero-agglutinin as separate substances from G. I, G. II, A. I, A. II and A. III; but it may well be that reductions in the number of chemically separate gamones will be effected in the future, though the actual list of functions may remain the same.

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* Individual papers have not been cited in Table 1. For these, reference should be made to Hartmann *et al.*, Runnström *et al.* and Tyler in the References.

Table 1

Modern name	Former name	Biological properties	Chemical characteristics
ynogamone I (G. I)	Fertilizin	Activates and/or stimulates spermatozoa; exerts chemotactic influence on spermatozoa	Heat resistant; claimed to be echinochrome, or to have echinochrome as prosthetic group, in *Arbacia pustulosa*
ynogamone II (G. II)	Fertilizin or iso-agglutinin	Specifically agglutinates homologous spermatozoa; derived from jelly round eggs, not from eggs	Protein; heat labile
—	Hetero-agglutinin	Non-specifically agglutinates spermatozoa	Protein (?)
ndrogamone I (A. I)	—	Paralyses spermatozoa and neutralizes G. I	Soluble in methanol; diffuses through cellophane; not inactivated by trypsin
ndrogamone II (A. II)	—	Dissolves or precipitates jelly round eggs; neutralises G. II.	Protein; properties somewhat similar to those of Hyaluronidase
ndrogamone III* (A. III)	—	Action similar to that of honey-bee venom and detergents on surface of sea-urchin eggs	Soluble in methanol; diffuses through cellophane; not inactivated by trypsin
ynandrogamone I (GA. I)	Antifertilizin	Combines with G. II; forms membrane on surface of jelly; obtained from eggs and spermatozoa	Protein; can be separated from A. II

* Also called Sperm Lysin (Runnström, 1946).

This paper is concerned with Androgamone I (A. I) which, according to Hartmann, Schartau & Wallenfels (1940), is responsible for spermatozoa being motionless in the testes of the sea-urchin, and for their senescence after they are mixed with sea water. Many explanations for the lack of sperm movement in non-mammalian semen have been put forward, though the variety of these explanations seems to have escaped the notice of several workers in this field. According to Hartmann *et al.* (1940), the hormone A. I is the responsible agent in the semen of *Arbacia pustulosa* and of the Rainbow Trout (Hartmann, 1944); the same conclusion was reached by Schartau & Montalenti (1941) in the case of the Lamprey, while von Medem (1945) appears to have confirmed the existence of A. I in certain Molluscs. No reference is made by Hartmann and his colleagues to an observation of Gray (1928) that sea-urchin spermatozoa are as motile in seminal plasma, obtained by centrifuging semen, as in sea water: nor is any reference made to the work of Schlenk, who concluded (at different times) that inactivation in trout semen is due to hydrogen ions, phosphate, or potassium ions (Schlenk, 1933; Schlenk & Kahmann, 1938). Runnström, Lindvall & Tiselius (1944) express some doubts about the role of A. I in sea-urchin semen, and tentatively suggest that the absence of movement in this case is due to CO_2 narcosis or O_2 lack. They assert, however, that in salmon semen A. I is responsible for sperm inactivation before dilution. Some support for Hartmann's views on the role of A. I in sea-urchin semen is found in the observations of Southwick (1939) on *Echinometra subangularis*. Southwick states that sperm of this species are made motionless by seminal plasma obtained by centrifugation. This result is in contradiction to that of Gray (1928) already mentioned, and those of Hayashi (1945, 1946), who also found that seminal plasma has no adverse effect on the viability and fertilizing power of spermatozoa of the same species of sea-urchin.

In this paper the cause of sperm immotility in sea-urchin semen is established. In a subsequent paper the alleged action of Androgamone I in inducing senescence of spermatozoa after dilution in sea water will be examined, together with certain allied problems.

MATERIAL

The semen of *Echinus esculentus* was obtained in the usual way (Gray, 1928).

METHOD

Gas chamber. Visual observations on semen were made in a gas-tight chamber (Fig. 1). The top of the chamber was sealed with a cover-slip, on the underside of which was a thin film of semen. The chamber was kept at a constant temperature by water circulating round but not through it. Different gases, such as commercial nitrogen, partially purified by bubbling through alkaline pyrogallol towers, could be passed through the chamber, as shown in Fig. 1.

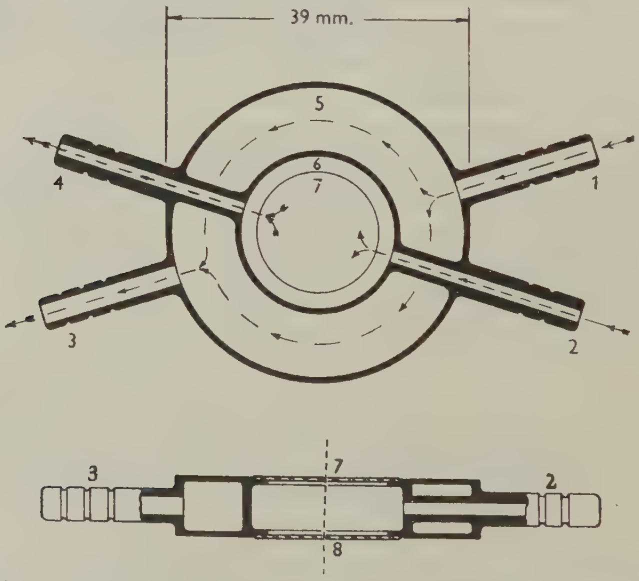

Fig. 1. Gas chamber. 1, circulating water inlet; 2, gas inlet; 3, water outlet; 4, gas outlet; 5, circulating water chamber; 6, support for cover-slip at top of gas chamber; 7, upper cover-slip; 8, lower cover-slip.

Oxygen electrode. The possibility of using platinum electrodes for measuring the O_2 tension in solution was first noted by Danneel (1897). Details of the theory of the Pt O_2 electrode and its application to biological material can be found in the papers of Davies & Brink (1942) and Hill (1948). The principle of the electrode, very briefly, is as follows: if a Pt electrode and some non-polarizable electrode, such as silver silver chloride wire, are immersed in an aqueous solution of an electrolyte, and a potential is applied between them, the Pt electrode being negative, the current through the system will remain almost constant over a fairly wide range of applied potential provided the solution is not too acid. The reaction at the Pt electrode (Hill, 1948) is probably

$$O_2 + 4H^+ + 4e \rightarrow 2H_2O.$$

The current remains constant in spite of changes in the applied potential because the supply of O_2 to the electrode is limited by the maximum rate at which O_2 can diffuse from the bulk of the solution to the electrode surface. The current which flows for a given potential across the electrode is therefore directly proportional to the O_2 tension in the solution. When the voltage across the system reaches a certain level, in the region of 0·8 V., hydrogen ions are reduced to molecular hydrogen at the electrode. This secondary reaction causes the current to rise again, and the system no longer functions as an O_2 electrode. There are two other conditions in which the system may not function as an O_2 electrode, and in which, therefore, the current will not be constant over wide ranges of applied voltage. These are first, if the solution whose O_2 tension is being measured is too acid, in which case hydrogen may be reduced at the Pt electrode; secondly, it would seem possible that there might be other substances in the solution which could react at the Pt electrode within the voltage range at which normally O_2 reacts according to the equation given above. These points are mentioned because some difficulties were experienced in the measurement of the O_2 tension of semen.

The current was measured by recording the fall of potential along a resistance in series with the potentiometer providing the e.m.f. across the electrodes. The potential across this resistance must be small compared with that across the electrodes, otherwise the latter will be too low and misleading results will be obtained. A resistance of 5 MΩ was found convenient for this purpose. The ohmic drop across this resistance was measured by a thermionic voltmeter (Rothschild, 1937) passing a grid current of less than 10^{-13} amp. With this apparatus it was possible to measure a current of about 10^{-11} amp. The recessed type of Pt electrode (100 μ diameter), prepared according to the method of Hill (1948), was used, the tip of the Pt wire being about 0·25 mm. within the end of the glass micro-pipette.

Measurements were made on semen in two ways: first, on a thick layer of semen in a specimen tube about 0·5 cm. in diameter, the electrodes being 0·5 cm. below the surface; secondly, on a thin film of semen on a microscope slide, to which a small drop of 40% formalin had been added to kill the sperm, and which had been equilibrated with air. These latter measurements determined the current flow in semen in which the normal physical barriers to diffusion of O_2 were obviated. In some cases bicarbonate was added to the semen to prevent the solution becoming too acid.

pH *measurements*. These were done with glass electrodes, calomel half cells through agar-sea water bridges, and the thermionic voltmeter already mentioned.

Potassium estimations. Mr R. Milton carried these out by a method involving the oxidation of organic material in the seminal plasma and subsequent determination of the cobaltinitrite (Milton, Hoskins & Jackmann, 1944).

RESULTS

Properties of seminal plasma. Eight determinations of the potassium content of seminal plasma from different sea-urchins were made. The results, in mg./ml., were: 2·18, 1·35, 1·14, 0·84, 1·65, 1·72, 1·78, and 1·75, the average value being 1·55 mg./ml. There is, therefore, considerably more K in seminal plasma than in sea water or *E. esculentus* perivisceral fluid, in both of which the concentration of K is 0·375 mg./g. water (Robertson, 1939). The high K content of seminal plasma is not, however, responsible for the lack of sperm movement in this medium, as will be shown later.

The pH of *E. esculentus* semen is between 7·3 and 7·7. The hydrogen ion concentration is not responsible for the lack of movement for the same reasons that apply in the case of K. Apart from this, sea-urchin spermatozoa are active at lower pH's than 7·3.

If semen is gently centrifuged, at 1500 r.p.m. (12 cm. radius), for about 15 min., after which the sperm and seminal plasma separate into distinct layers, and a drop of semen from another sea-urchin is placed in this seminal plasma, the spermatozoa are as active as in sea water. This experiment and the one in the next section dispose of the possibility that potassium or hydrogen ions are responsible for the lack of sperm motility in semen; it also shows that no substance, such as A.I, normally diffuses out of sea-urchin spermatozoa and prevents them moving. However, a substance which does inhibit movement can be separated from spermatozoa by prolonged or more violent centrifugation; its action is unaffected by oxygenation of seminal plasma. There is little doubt that this substance is A.I. The ease with which A.I can be displaced from spermatozoa into the seminal plasma varies greatly from urchin to urchin. This probably explains the conflicting results obtained by Gray (1928), Hayashi (1945, 1946) and Southwick (1939) on the effect of seminal plasma obtained by centrifugation on the motility of spermatozoa of the same species.

While examining the displacement of A.I from spermatozoa into seminal plasma I noticed that the latter has quite a strong catalatic activity, estimated by the method of Bonnichsen, Chance and Theorell (1947); it is about equal to that of human blood diluted 1:50. This catalatic activity is powerfully inhibited by hydroxylamine hydrochloride. The presence of catalase in semen and seminal plasma is interesting as mammalian spermatozoa contain insignificant amounts of this enzyme (Blom & Christensen, 1947), but it has not yet been proved that the catalase in sea-urchin semen is derived from the spermatozoa.

Oxygenation of semen. When a drop of semen is placed in the gas chamber in air, the spermatozoa will be found to be motionless. Sometimes a few spermatozoa are feebly motile at the edges of the drop, as Runnström *et al.* (1944) observed, but this is not always the case. If, after establishing that spermatozoa do not move, O_2, unsaturated with water vapour, is introduced into the gas chamber, the spermatozoa in the drop of semen start moving. The translatory movements are slow, presumably because the spermatozoa are so tightly packed. When examining semen in O_2 it is advisable to flush the gas chamber out with O_2, seal off the inlet and outlet tubes, and examine the sample after the gas flow has ceased. This obviates the possibility of the sperm movements being due to convection or other disturbances caused by the flow of O_2 over the surface of the drop. When the spermatozoa in the drop of semen are clearly motile, N_2 is passed through the gas chamber for a few minutes and the chamber is again sealed off. No precautions to remove the last traces of O_2 from the nitrogen are necessary, as, apart from the rapidity of O_2 diffusion out of so small a drop, the spermatozoa in the semen themselves consume any residual O_2 that may be left. In an atmosphere of N_2 the sperm stop moving.

The introduction of N_2 into the gas chamber before the spermatozoa have been activated by O_2 does not cause any movement. This fact disposes of the possibility that activation is due to the removal of CO_2 from the semen, a suggestion put forward by Runnström *et al.* (1944).

When activated spermatozoa are made motionless by the admission of N_2, the reintroduction of O_2 makes them motile again. Alternating atmospheres of O_2 and N_2 produce alternating periods of movement and inactivity: the process can be repeated frequently.

Oxygen tension in semen. Satisfactory current-voltage curves were obtained with sea water equilibrated with air and with N_2. That is to say, the current remained constant (in the case of sea water equilibrated with N_2, zero) over wide ranges of applied potential. In the case of semen, however, difficulties were experienced. In three out of twenty determinations the current through the semen was zero up to approx. 0·8 V., showing that the O_2 tension was zero. In other cases, however, the current through the semen, though markedly lower than that through the thin film of formalin-treated semen on the microscope slide, was not zero and was about 0·2 of that through the latter, suggesting an O_2 tension of some 30 mm. Hg. In these cases the current was not constant when the potential was varied, and polarization occurred at too low an applied voltage. The same occurred with the formalin-treated drop. The unsatisfactory plateau obtained suggests either that hydrogen ions are reduced to molecular hydrogen at a lower potential than in an alkaline aqueous electrolyte, or that some other unknown substance is reduced at the electrode surface. Although the pH of semen in which the sperm were alive was found to be 7·5, this might not be so in the thin film in which the spermatozoa had been killed by formalin; the thin film and the bulk semen were, therefore, buffered with bicarbonate to obviate this possibility. Fig. 2 is an example of the type of curve obtained in these conditions. The addition of bicarbonate (previously in contact with air) might tend to increase the O_2 tension in the case of bulk semen, so that this alteration in the experimental conditions is unfavourable from the point of view of finding low O_2 tensions. The current flowing between the electrodes in bicarbonate-treated semen was found to be 0·1 of that in similar semen in which the sperm had been killed by formalin and which had been equilibrated with air. This means that the O_2 tension is approx. 15 mm. Hg in semen as compared with 153 mm. Hg in air.

The results are unsatisfactory in the sense that they do not dispose of the possibility that some other substance apart from O_2 is reacting at the electrode surface. The material is not perhaps ideal for measurements of O_2 tensions as it has the consistency of thick cream, and the seminal plasma may contain a number of unknown organic substances which might interfere with the O_2 tension determination.

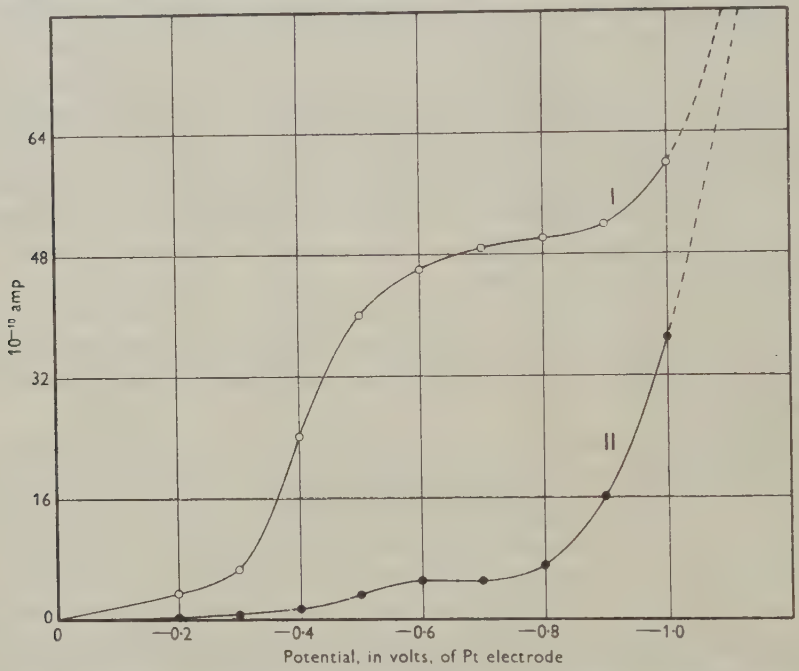

Fig. 2. Current-voltage curves for *Echinus esculentus* semen in a thin film equilibrated with air, I, and in a thick layer, II. Between 0·6 and 0·8 V., where the rate of change of current with voltage is small, the current is approximately proportional to the O_2 tension.

DISCUSSION

The experiments show that *E. esculentus* spermatozoa do not move in undiluted semen because of the lack of O_2. There is about four times as much K in the seminal plasma as in the perivisceral fluid or sea water; the pH of semen is somewhat lower than that of sea water, and centrifugation of semen may cause toxic substances to be released from the spermatozoa. These facts have no bearing on the normal causes of sperm immotility. This result is not unexpected as diluted sea-urchin spermatozoa, unlike those of mammals, cannot move in the absence of O_2 (Barron, 1932). This means that the metabolic processes producing the energy needed for movement must be different from those occurring in mammalian spermatozoa. This is confirmed by MacLeod's observation (1941) that cyanide, azide, and carbon monoxide have no inhibitory action on human spermatozoa, though cyanide and carbon monoxide reversibly inhibit the respiration, and to a certain extent the motility, of sea-urchin spermatozoa (Robbie, 1948; Rothschild, 1948). One can conclude that the cytochrome system is more directly concerned with the chemical changes which produce the energy for contraction in sea-urchin spermatozoa than in mammalian spermatozoa.

On the other hand, the fact that sea-urchin spermatozoa are alive, but motionless, in semen, and that anaerobiosis, though inhibiting motility, does not irreversibly injure diluted spermatozoa for considerable periods of time (Harvey, 1930), shows that the sea-urchin spermatozoon contains a metabolic system which is capable of functioning to a certain extent under anaerobic conditions.

Runnström *et al.* (1944) state categorically that Androgamone I is responsible for the lack of movement of salmon spermatozoa in a 'dense suspension' (p. 285). The same views have been expressed about the semen of various lower organisms, as was mentioned in the Introduction. The possibility of extracting inhibitory substances from spermatozoa is not in dispute. But as such substances are not normally responsible for the lack of sperm movement in sea-urchin semen, it would be important to see whether salmon and trout spermatozoa in semen become active when the O_2 tension is increased. Until such experiments are done, it would perhaps be unwise to assign too important a role in gamete physiology to the unidentified and unspecific Androgamone I.

The possibility that the seminal plasma of sea-urchins contains a substance whose inhibitory action is neutralized by O_2 may be worth mentioning, but it seems unlikely that so remarkable a property would have escaped attention during examination of Androgamone I. In any case, it has been established that oxygenation does not destroy the inhibitory substance found in seminal plasma after centrifugation.

The initiation of sperm movement in semen by O_2 and its subsequent inhibition by N_2 show without reasonable doubt that O_2 lack is the cause of sperm immotility in undiluted semen. It has not, however, been possible to show that the O_2 tension in semen is as low as this result might lead one to expect. If it had been possible to establish that the O_2 tension in semen is 15 mm. Hg, the controversial question would arise as to whether a critical O_2 tension is necessary for the initial activation of spermatozoa. This cannot be considered, as the apparent residual O_2 tension in undiluted semen may not be due to the presence of O_2, but to some unidentified substance reacting at the electrode surface and causing a flow of current in spite of the comparative absence of O_2.

SUMMARY

1. *Echinus esculentus* spermatozoa are normally motionless in undiluted semen. They become active when the semen is diluted with sea water or seminal plasma obtained by gentle centrifugation (1500 r.p.m., 12 cm. radius, for 15 min.).
2. A sperm-immobilizing substance can, however, be obtained in seminal plasma by more prolonged centrifugation of semen.
3. Spermatozoa can be made motile in undiluted semen by increasing the O_2 tension in the atmosphere surrounding the semen.

4. This O_2 activation is completely inhibited by N_2; the N_2 effect is reversible.

5. Measurements of seminal O_2 tension were made with an O_2 electrode. The O_2 tension of semen is low, being at most 15 mm. Hg. It was not possible to decide whether this residual tension was due to O_2 or some other substance reacting at the electrode.

6. The K content of seminal plasma is about 1·55 mg./ml., which is four times higher than that of perivisceral fluid or sea water.

7. The pH of semen is lower than that of sea water, being approx. 7·5.

8. Neither K concentration nor pH is responsible for the inactivity of sperm in semen.

9. A hormone, Androgamone I, is often considered to be responsible for the inactivity of spermatozoa in non-mammalian semen. No support has been found for this view and it is concluded that in *E. esculentus* semen the spermatozoa are motionless through lack of O_2.

I am much obliged to the Director and Staff of the Scottish Marine Biological Station, Millport, where the experiments were carried out.

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THE PHYSIOLOGY OF SEA-URCHIN SPERMATOZOA

SENESCENCE AND THE DILUTION EFFECT

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(With Five Text-figures)

INTRODUCTION

Hartmann, Schartau & Wallenfels (1940) suggested that the senescence of sea-urchin spermatozoa was due to the action of Androgamone I (A.I).* If this implies that senescence is caused by the accumulation of some toxic product of metabolism within the sperm, it is another way of stating a hypothesis put forward by Gray (1928*b*) and called by him a theory of intoxication. If A. I diffuses out of spermatozoa and exerts its toxic influence from outside, it might be chemically or biologically identifiable in the medium. This latter possibility has been the subject of experiments, described in this paper.

An important biological fact to be taken into consideration when studying the senescence problem is that dilute suspensions of spermatozoa move and respire at a higher rate, and consume more oxygen during their active life, than more concentrated suspensions (Gray, 1928*a*). This is called the Dilution Effect. Gray suggested that the lack of movement in semen and the reduced movement and respiration in concentrated suspensions was due to allelostasis—physical constraint resulting from the proximity of the spermatozoa. This view requires modification, as does Hartmann's, that A. I is the responsible agent in both cases (Rothschild, 1949). Runnström, Tiselius & Lindvall (1945, p. 21) state that 'the carbon dioxide content of dense suspensions† probably is a sufficient inhibitor of the motility of the sperm'. This suggestion is difficult to reconcile with the well-established fact that concentrated and dilute sperm suspensions respire at different rates in manometric experiments in which CO_2 is continuously removed by caustic potash.

Senescence may be due to depletion of intracellular substrates, to auto-intoxication, or to the exhaustion of some essential unit in the metabolic cycle. As regards the first of these alternatives, Goldinger & Barron (1946) observed that the O_2 uptake of sea-urchin spermatozoa increased by 30% after addition of succinate to the medium. Although this, and the observation that O_2 uptake is photo-reversibly inhibited by CO (Rothschild, 1948), show that the respiration of sea-urchin spermatozoa is mediated through the succinoxidase system, the succinate in

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* 'Durch den spermalähmungsstoff werden die Spermien in den Gonaden und beim längeren Verweilen in Seewasser in ihren Bewegungen gehemmt' (p. 417). The 'sperm-paralysing' substance makes the sperm motionless both in the testes and after having been in sea water for some time.

† Of *Strongylocentrotus droebachiensis* and *Echinocardium cordatum* sperm.

Goldinger & Barron's experiments may not have been utilized directly as a source of energy. There is a possibility that it was acting catalytically as a hydrogen carrier, in a Szent-Györgyi or Krebs cycle. The resolution of this question requires measurements of the R.Q. and of changes in O_2 uptake when different amounts of succinate are added to the medium. Alternatively, the succinate oxidation may have had nothing to do with the normal metabolism of the cell. Other substances increase the O_2 uptake of sea-urchin spermatozoa when added to sea water; of these, echinochrome and similar substituted naphthoquinones (Hartmann & Schartau, 1939), or thyroxine (Carter, 1932), can hardly be considered as substrates. Propyl and cynnamyl alcohol (Clowes & Bachman, 1921) might come into this category, while a new sperm activator, ethanol, will be discussed in a later paper. The auto-intoxication explanation of senescence has only been investigated by Gray's indirect analytical method (1928*a*). According to Gray's theory, a linear relationship between total oxygen consumed and the square root of the duration of the experiment indicates auto-intoxication. Gray (1928*a*) and Hayashi (1946) found that such a linear relationship does exist, but as Gray (1931) pointed out, the results obtained by indirect analytical methods are difficult to interpret with certainty.

In this paper a further examination is made of Senescence and the Dilution Effect. The experiments were designed to establish correlations between the observed biological phenomena and the physiological condition of the material. The methods of investigation were to measure the effect of the following substances or changes in environmental conditions on sea-urchin spermatozoa:

Supernatant sea water from metabolizing sperm suspensions.
Sea water at different pH's.
Variations in O_2 tension on suspensions containing different quantities of sperm.
CO on suspensions containing different quantities of sperm.
Motility of spermatozoa in the presence of CO.

The possibility of sea-urchin spermatozoa glycolysing was also investigated. Finally, the content of oxidizable substrate was examined after motility and respiration had ceased.

MATERIAL

The spermatozoa of *Echinus esculentus*, obtained in the usual way, were suspended in sea water. The semen was not washed before dilution.

METHOD

O_2 *uptake*. Respirometric experiments were carried out in standard Barcroft-Warburg manometers, as described in a previous paper (Rothschild, 1948). Details will be found in Table 1. The sign '♂' means 'spermatozoa' in tables.

Glycolysis. Barcroft-Warburg manometers without KOH in the centre cups, with N_2/CO_2 or O_2/CO mixtures in the gas phase, and manometric estimations of bicarbonate.

Dehydrogenase activity. Thunberg tubes (see text).

Concentration of sperm. The original suspensions were diluted a known number of times and the spermatozoa killed with a drop of 40% formalin. Counts were then made, accurate to ± 10%, with a Fuchs-Rosenthal haemocytometer. Dark-ground illumination was found to be convenient, as the sperm tails show up brilliantly with this form of lighting.

Some workers have tried to make their respirometric measurements quantitative by estimating the amount of nitrogen in the suspensions. As sea-urchin seminal plasma contains significant amounts of protein, the nitrogen found in suspensions is not entirely derived from the spermatozoa. Furthermore, the relative concentrations of sperm and seminal plasma vary over wide limits in different sea-urchins, and the amount of seminal plasma introduced into the suspension depends on the density of sperm required for the experiment.

Sperm motility. There is at present no convenient and objective method of assessing sperm motility. Recently a graph of sperm movement under different environmental conditions was published (Robbie, 1948). This was based on visual observation (personal communication), and the graph is unquantitative. The visual observations recorded later in this paper were carried out on sperm in a gas chamber which has been previously described (Rothschild, 1949). Their object was to determine whether sperm movement was stopped or slowed down by CO. The gas mixture used was 5% O_2 in CO and 5% O_2 in N_2 for the controls. The O_2 uptake of sea-urchin spermatozoa is strongly inhibited by CO, the inhibition being reversed by light (Rothschild, 1948). To study the inhibition of movement by CO, the wave-length of the light used for visual observation under the microscope must be outside the regions of photochemical absorption by CO-cytochrome oxidase (see Fig. 5*b*). A Farrand Interference filter, transmitting light of wave-length 548 mμ, with a half-band width of 36 mμ and a 36% transmission, which fulfils these conditions, was therefore interposed between the light source and the microscope mirror. The spermatozoa could then be observed in the presence of CO as if they were in the dark. The wave-length of the transmitted light was checked spectroscopically, and it was noticed that unless the filter was normal to the incident beam, other wave-lengths than 548 mμ were transmitted. This is a characteristic of these Interference filters, which makes careful adjustment essential.

pH *measurements*. Glass electrode, calomel half-cell through agar-sea-water bridge and thermionic voltmeter.

Centrifuge. 1500 r.p.m., 12 cm. radius.

RESULTS

Extracellular Androgamone I. The object of these experiments was to find out if any inhibitory substance diffuses out of spermatozoa and reduces motility and respiration. A suspension containing about 10^9 sperm/ml. was allowed to metabolize in a beaker, without agitation, for 2 hr. The suspension was then centrifuged and the supernatant sea water so obtained was used as the dilution medium for spermatozoa from another sea-urchin in a manometric experiment. The control suspension was made up with spermatozoa from the second sea-urchin and ordinary sea water whose pH had been brought down with HCl from 8·00 to 7·40, the pH of the supernatant sea water. (If the reverse procedure is attempted with NaOH, $Ca(OH)_2$ and $Mg(OH)_2$ are precipitated.) The concentration of sperm was the same in the experimental and control vessels, being approximately 10^8 sperm/ml. Fig. 1 shows the result of such an experiment, done in duplicate. So far from having an inhibitory effect on respiration, the supernatant sea water has a small but detectable protective action in maintaining the respiration of the spermatozoa. No difference in motility could be observed in the two media. As the original sperm suspension was rather strong, the results might have been due to a protective influence of seminal plasma brought into the sea water with the spermatozoa (Hayashi, 1946). There might therefore have been an antagonistic effect between seminal plasma and A. I, although Hartmann *et al.* (1940) believe that A. I is also present in seminal plasma. This possibility can be dismissed as the effect is slightly more marked if the sperm suspension from which the supernatant sea water is obtained is shaken before use. This suggests that the increased O_2 consumption is caused by a substance derived from the spermatozoa and not from the small amount of seminal plasma carried over from the semen.

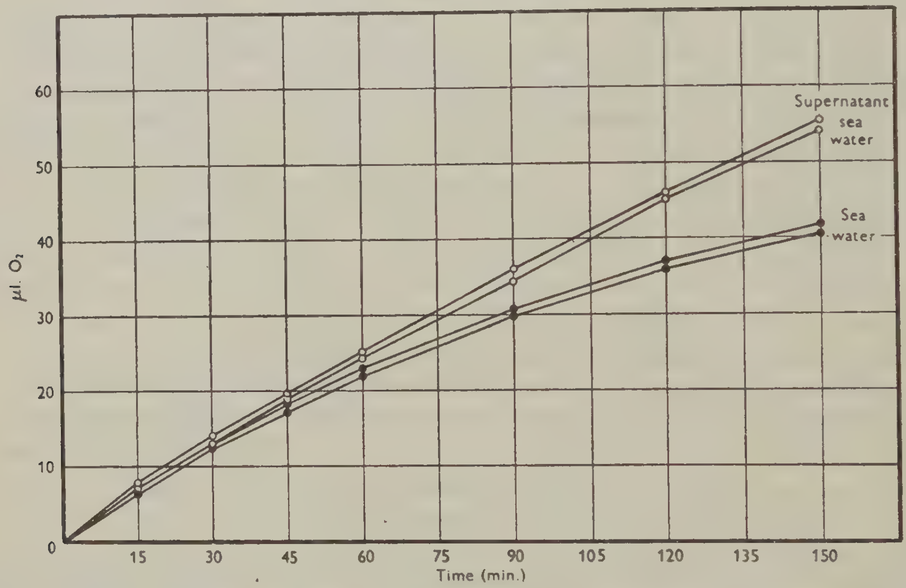

Fig. 1. Effect of supernatant sea water on O_2 consumption of spermatozoa. Ordinates, μl. O_2 consumed; abscissae, time in minutes. $T°$ C., 16·00; shaker speed, 96 c.p.m.; pH of supernatant sea water and control sea water, 7·40.

These results, which indicate that the hypothetical extracellular A. I plays no part in senescence, might have been due to the O_2 uptake of the control spermatozoa being so subnormal, through acidification of the medium, as to mask any difference between the respiration of the control and experimental suspensions. To test this possibility an examination was made of the O_2 uptake of identical suspensions at different pH's, the values being 7·78, 6·80, 5·90 and 5·00. Before the experiment, the spermatozoa in pH 5·00 sea water had agglutinated into macroscopic clusters and were only feebly motile, but after 90 min. in the manometer the difference between their respiration and that of sperm in sea water at pH 7·78 was negligible, while the pH had nearly risen to the control value. Examination at the end of the experiment revealed that the clusters had disappeared and that the spermatozoa were normal. The same applied to the respiration of the other suspensions in initially acidified sea water. This experiment disposes of the possibility that pH changes are responsible for the Dilution Effect. The same conclusion can be reached in a different way. The bicarbonate content of a sperm suspension can be measured by adding acid to the suspension and noting the positive CO_2 pressure in a manometer which contains no KOH. If the bicarbonate contents of two identical sperm suspensions are measured in this way, in one case at the beginning, and in the other at the end of the experimental period, no difference in bicarbonate content is found. This proves that there is no aerobic glycolysis during sperm metabolism.

E. esculentus spermatozoa do not glycolyse in anaerobic conditions whether isotonic dextrose (final concentration 1 %) is present in the sea water or not. These measurements were carried out in manometers without KOH in the centre cups; in these circumstances a positive pressure would have been caused by glycolysis and none was observed. The same applied when the gas phase contained $CO/CO_2/O_2$ mixtures.

CO_2 *inhibition*. Although the continuous removal of CO_2 by KOH in respirometric experiments would seem to preclude the possibility of the presence or absence of CO_2 having any bearing on the Dilution Effect, Runnström *et al.* (1945) have revived Cohn's original view (1918) that CO_2 has a narcotic effect when present in sufficient quantities, i.e. in dense suspensions. At the shaking rates used, 96 c.p.m., it was found that the KOH in the centre cups of the manometer vessels quickly absorbed much larger quantities of CO_2, produced suddenly in the main vessels, than those evolved by the spermatozoa. Dilution of CO_2 in solution therefore cannot be responsible for the Dilution Effect observed in manometric experiments. The experiments previously described on the acidification of sperm suspensions show that even if the evolution of CO_2 causes a fall in the pH of the suspension *in vivo*, this has no bearing on the fact that dense suspensions consume less oxygen than dilute ones, in manometric experiments.

Variations in O_2 *tension*. As motionless sperm in semen can be made motile by an increase in O_2 tension (Rothschild, 1949), it is natural to consider whether the Dilution Effect is influenced by changes in O_2 tension. Fig. 2 shows the results of a simple experiment to test the effect of varying O_2 tensions on sperm respiration, the O_2 uptake being measured in the presence of 100% O_2, 20% O_2 in N_2, and 10% O_2 in N_2. Each manometer flask contained 3·0 ml. of a rather concentrated sperm suspension, $2{\cdot}88 \times 10^9$ sperm/ml. Ten minutes' equilibration was allowed and the shaker speed was 100 c.p.m. with a 4 cm. stroke. After the 30 min. reading, the O_2 uptake of the suspension in equilibrium with O_2 was approximately 1·7 that of the suspension with 10% O_2 in the gas space; this difference was not maintained and at the end of the experiment, 105 min., the ratio had fallen to about 1·5. The experiment raises two questions: first, why does an increase in O_2 tension increase the O_2 uptake? Secondly, why is the effect of increased O_2 tension not maintained? The answer to the first question will be deferred. The answer to the second is found in the experiment described in Table 1. Columns 3, 15, 16 and 17 show that an increased O_2 tension increases O_2 uptake at the beginning of the experiment, but later on causes a definite inhibition of respiration. Pure oxygen, therefore, has a toxic action on sea-urchin spermatozoa, a phenomenon which has been observed in other tissues (Dickens, 1946*a*, *b*; Mann & Quastel, 1946). The experiments in Table 1 are an extension of those in Fig. 2, in that the O_2 uptake of various concentrations of spermatozoa at different O_2 tensions is compared. In these experimental conditions the toxic action of high O_2 tensions becomes very marked at low sperm concentrations. Columns 18 and 19 show that at sufficiently low sperm concentrations, pure oxygen depresses respiration from the start of the experiment, though there may of course have been an increase in O_2 uptake during the equilibration period.

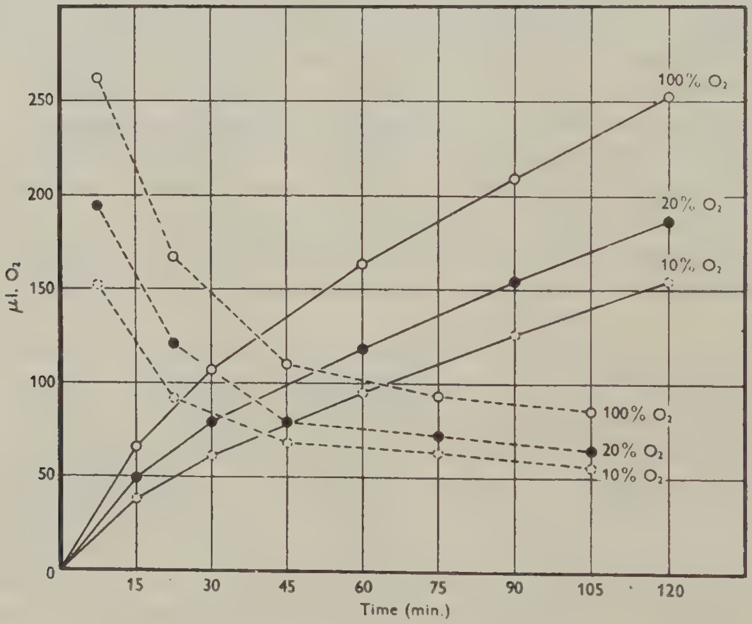

Fig. 2. O_2 uptake of a suspension containing $2{\cdot}9 \times 10^9$ sperm/ml., in equilibrium with different O_2 tensions. Ordinates, μl. O_2 consumed; abscissae, time in minutes. The broken curves are rates of O_2 uptake/hr., plotted on the same scale as the cumulative curves. $T°$ C., 16·00; shaker speed, 100 c.p.m.

Fig. 3 is a record of the O_2 uptake of different concentrations of sperm in sea water at varying O_2 tensions (100, 20 and 5% O_2), the dilutions being made in the manometer vessels, after equilibration. Before dilution, which was effected by tipping at 15 min., there is the characteristic increase in O_2 uptake at high O_2

Table 1

Material: *E. esculentus* spermatozoa.
Fluid: Sea water.
Gas: 100 % O_2 and 5 % O_2 in N_2.

Temperature: 16·0° C.
Shaker: 100 c.p.m.; stroke, 4·0 cm.
Equilibration time: 10 min.

Vessel no.	*Tb*	3	15	16	17	18	19
kO_2		1·243	1·262	1·266	1·287	1·330	1·228
Contents	3·2 ml. dist. H_2O	3 ml. sea water 1·7 × 10^9 ♂ 0·2 ml. 10 % KOH Filter-paper 5 % O_2 in N_2	3 ml. sea water 1·7 × 10^9 ♂ 0·2 ml. 10 % KOH Filter-paper 100 % O_2	3 ml. sea water 5·6 × 10^8 ♂ 0·2 ml. 10 % KOH Filter-paper 5 % O_2 in N_2	3 ml. sea water 1·9 × 10^8 ♂ 0·2 ml. 10 % KOH Filter-paper 100% O_2	3 ml. sea water 1·9 × 10^8 ♂ 0·2 ml. 10 % KOH Filter-paper 5 % O_2 in N_2	3 ml. sea water 1·9 × 10^8 ♂ 0·2 ml. 10 % KOH Filter-paper 100 % O_2
Scaling factor	—	1·243	1·262	3·798	3·861	11·970	11·052
0–60 min.	—	46·5 µl. O_2	54·2 µl. O_2	58·2 µl. O_2	62·9 µl. O_2	62·3 µl. O_2	51·9 µl. O_2
60–120	—	42·0	46·1	52·1	53·7	26·4	22·1
120–180	—	30·0	36·6	39·5	39·0	6·0	6·6
180–240	—	22·8	26·0	13·0	12·4	—	—
240–300	—	19·0	18·0	7·6	6·9	—	—
300–360	—	17·8	14·4	—	—	—	—

Table 3

Material: *E. esculentus* spermatozoa.
Fluid: Sea water.
Gas: 10 % O_2 in CO and 10 % O_2 in N_2.

Temperature: 16·0° C.
Shaker: 96 c.p.m.; stroke, 4·0 cm.
Equilibration time: 10 min.

Vessel no.	3	16	4	17	15	18
kO_2	1·243	1·266	1·243	1·287	1·262	1·330
Contents	3 ml. sea water 1·5 × 10^8 ♂ 0·2 ml. 10 % KOH Filter-paper 10 % O_2 in CO	3 ml. sea water 1·5 × 10^8 ♂ 0·2 ml. 10 % KOH Filter-paper 10 % O_2 in N_2	3 ml. sea water 7·5 × 10^7 ♂ 0·2 ml. 10 % KOH Filter-paper 10 % O_2 in CO	3 ml. sea water 7·5 × 10^7 ♂ 0·2 ml. 10 % KOH Filter-paper 10 % O_2 in N_2	3 ml. sea water 3·8 × 10^7 ♂ 0·2 ml. 10 % KOH Filter-paper 10 % O_2 in CO	3 ml. sea water 3·8 × 10^7 ♂ 0·2 ml. 10 % KOH Filter-paper 10 % O_2 in N_2
Dark–0–20 min.	6·1 µl. O_2	10·9 µl. O_2	6·3 µl. O_2	14·5 µl. O_2	5·1 µl. O_2	12·5 µl. O_2
Ratio of control O_2/inhibited O_2	1·8		2·3		2·5	

tension, the respiration in O_2 being nearly double that in 5% O_2. After dilution (1/7·5), however, high O_2 tensions have no stimulating effect on respiration. Gas equilibration is unlikely to be so satisfactory in the side bulbs as in the main vessels, in spite of the relatively high shaking speed, and accounts for part at any rate of the difference between the suspensions before dilution. The toxic action of pure oxygen is usually observed towards the end of the life of the suspension.

These experiments do not establish the existence of physiological differences between sperm in dense and in dilute suspensions. The possibility that the observed differences in respiration were due to diffusion limitations was investigated by examining the O_2 uptake of dense sperm suspensions with different O_2/N_2 mixtures in the gas phase, and at different manometric shaking rates, the experiments being done simultaneously. The results are shown in Table 2.

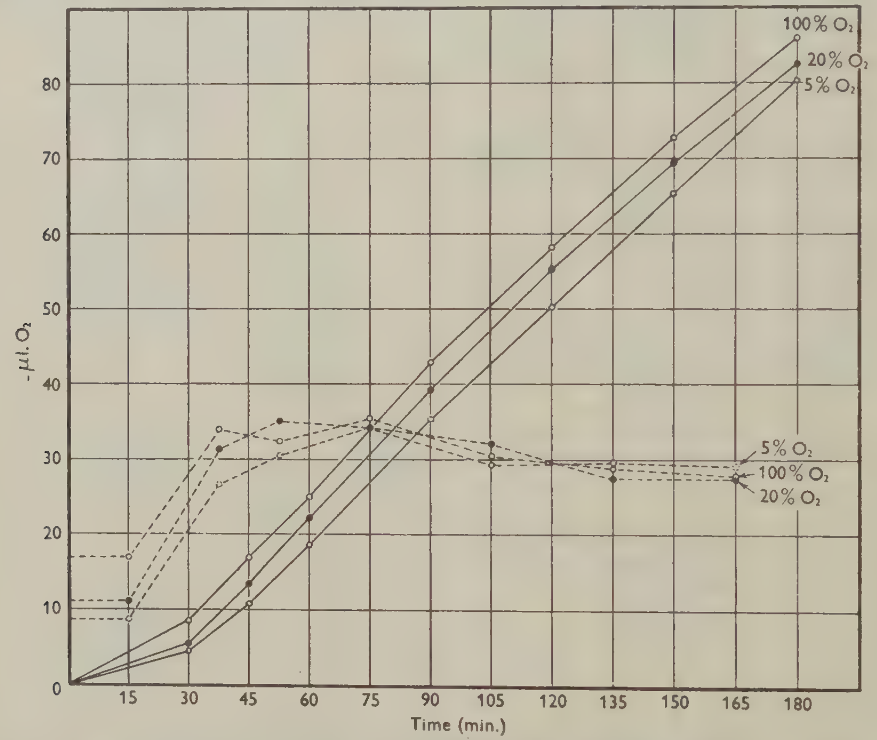

Fig. 3. O_2 uptake of suspensions, initially containing $2·8 \times 10^8$ sperm/ml., after 7·5:1 dilution with sea water, at different O_2 tensions. Ordinates, μl. O_2 consumed; abscissae, time in minutes. The broken curves are rates of O_2 uptake/hr., plotted on the same scale as the cumulative curves. $T°$ C., 16·00; shaker speed, 100 c.p.m.

The table shows that at the higher shaking speed there was no difference between the O_2 uptake in the presence of 5% O_2 in N_2 and 100% O_2. At the lower shaking speed the difference was 33%. Comparison of respiration in 5% O_2 in N_2 at the two shaking speeds shows that O_2 uptake is 47% lower when the shaking speed is 96 c.p.m. Shaker speeds above or on occasions as high as 120 c.p.m. tend to damage the sperm, with reduction of O_2 uptake; it is therefore impossible to establish manometrically that adequate O_2 saturation occurs in dense sperm suspensions at 120 c.p.m.

Table 2

Material: *P. miliaris* spermatozoa.
Fluid: Sea water.
Gas: 100% O_2, 20% O_2 in N_2, and 5% O_2 in N_2.

Temperature: 16° C.
Shaker: 96 c.p.m. and 120 c.p.m.
Equilibration time: 10 min.

	Shaker, 120 c.p.m.		Shaker, 96 c.p.m.		
Gas	5% O_2 in N_2	100% O_2	5% O_2 in N_2	20% O_2 in N_2	100% O_2
µl. O_2/hr.	99	100	67	86	89

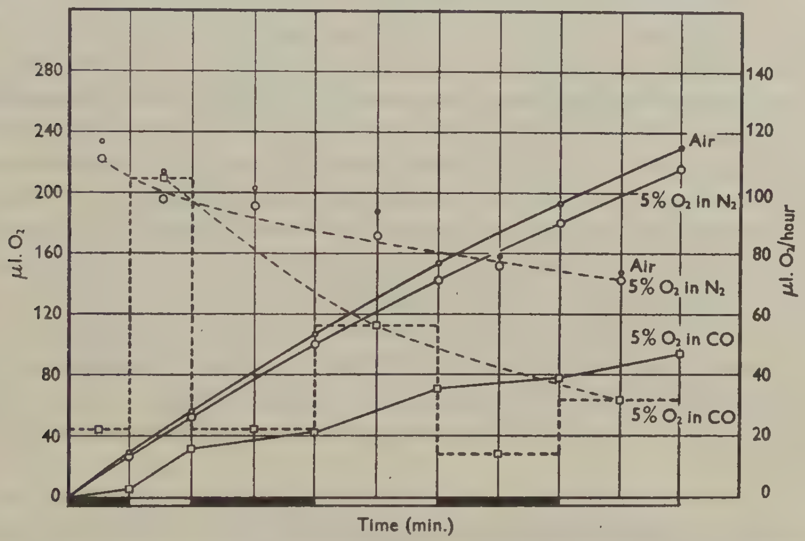

Fig. 4. O_2 uptake of a suspension containing $3\cdot0 \times 10^8$ sperm/ml. equilibrated with air, 5% O_2 in N_2, and 5% O_2 in CO. Ordinates, left, µl. O_2 consumed; ordinates, right, µl. O_2 consumed per hour; abscissae, time in minutes. Dark and illuminated periods are indicated by black and white lines under the base line. The broken rectangular curve represents the average O_2 uptake/hr. of the CO/O_2 suspension. The corresponding curves for the air and N_2/O_2 suspensions are omitted for the sake of clarity. The 'illuminated' points in the CO/O_2 curve are joined by a broken curve for comparison with the N_2/O_2 rate curve. *T*° C., 16·00; shaker speed, 96 c.p.m.

Action of CO *on different concentrations of sperm.* In the same way as different O_2 tensions have different effects on O_2 uptake according to the sperm concentration, CO inhibition varies with the density of the suspension. CO has a greater inhibitory action on the O_2 uptake of dilute than on concentrated suspensions (Table 3, see p. 359).

Examination of previous results (Rothschild, 1948) suggests that CO may have an irreversible toxic action on sea-urchin spermatozoa, in addition to photo-reversible inhibition of respiration. These previous experiments were done with 10% O_2 in CO in the gas space, in which conditions the irreversible toxic action is barely visible during the first 2 hr. of the experiment. If the CO concentration is increased to 95%, the decline in O_2 uptake during periods of illumination is very marked. In Fig. 4 the O_2 uptake/hr. of the 5% O_2 in N_2 control is 1·5 times that of the experimental suspension during the second period of illumination. For the next period of illumination the factor is 2·3. This is not caused by incomplete photo-reversal of CO inhibition, as in the first period of illumination, the O_2 uptake of the experimental suspension is actually higher than that of the control. This also disposes of the possibility that the difference is caused by hysteresis, or a time lag, before illumination achieves reversal. The increased O_2 uptake of the illuminated experimental vessel, as compared with the control, which is unaffected by illumination, is a characteristic feature of the early phases of CO experiments.

Fig. 5*a* shows the results of illuminating a CO-inhibited suspension with different wave-lengths of light. In the control the gas mixture in equilibrium with the suspension was kept in the dark for 15 min., illuminated with 548 mμ light for the next 15 min., and then illuminated with white light (carbon arc without filter) for a further 15 min. Fig. 5*a* shows a 70% inhibition of O_2 uptake in the dark and no change in respiration on illumination with 548 mμ light. Furthermore, the inhibition is completely reversed by white light. The white light is of course more intense than the 548 mμ light, because the Interference filter only transmits 36% of the light from the arc. But it is easy to show that the non-reversal of inhibition in 548 mμ light is due to there being no absorption in this region of the spectrum and not to the reduced illumination, by replacing the 548 mμ filter by one transmitting 420 mμ light of the same intensity. Complete reversal of inhibition occurs in these conditions. The positions of 420 and 548 mμ light relative to the photochemical absorption spectrum of CO cytochrome oxidase are shown in Fig. 5*b*.

For visual observation, the same suspension, or one containing the same concentration of spermatozoa, was transferred into the gas chamber under the microscope, the same light source and 548 mμ light filter being used. As mentioned earlier, accurate visual estimations of small changes in sperm motility are difficult to make. Moreover, observations with light of different wave-lengths, especially 548 mμ light, tend to increase these difficulties because the number of spermatozoa visible in the field is less than with white light. A superficial examination may therefore lead to the conclusion that there is a reduction in movement when the filter is switched into the beam from the arc light. If, however, individual spermatozoa, or particles which are agitated by the spermatozoa, are observed while the change from 548 mμ to white light is effected, it can definitely be established that the decrease in motility is far less than the decrease in respiration. A 70% reduction in motility corresponding to the decrease in O_2 uptake would be easily recognizable: three independent observers were unable to detect any reduction in motility of this order.

Intracellular substrate after cessation of respiration and motility. 3 ml. of a 10^8/ml. sperm suspension in sea water are placed in a Thunberg tube with 0·3 ml. 1/10,000 methylene blue in sea water in the stopper. On mixing after evacuation, the methylene blue is completely reduced in 20 min. at 16° C. No reduction takes place if the sperm suspension is previously kept at 60° C. for 15 min. This shows that the reduction of the methylene blue is not due to 'chemical' reducing agents in the suspension, but to dehydrogenases within the sperm. Furthermore, dehydrogenases would be unable to reduce the methylene blue if no substrates were available within the sperm.

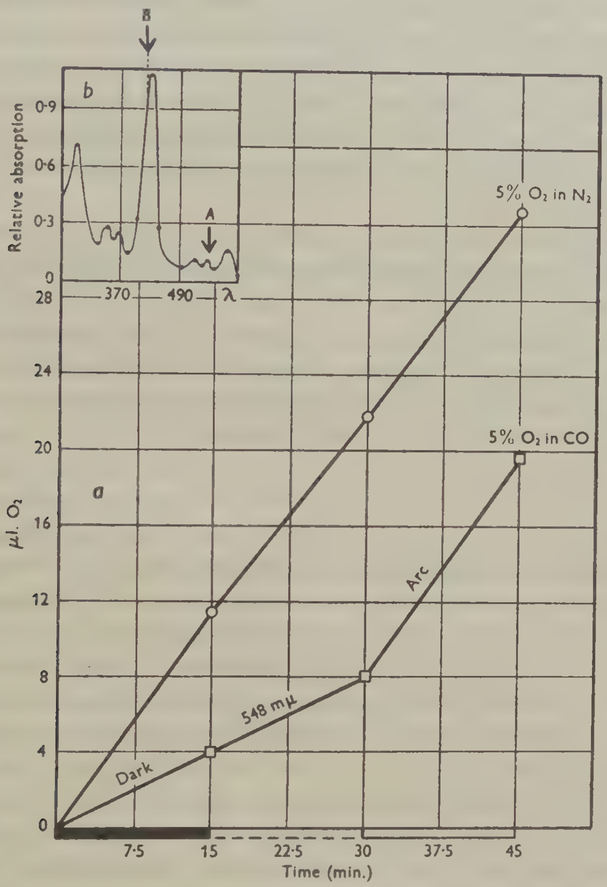

Fig. 5. *a*. O_2 uptake of two identical sperm suspensions, one equilibrated with 5% O_2 in N_2, the other with 5% O_2 in CO. Ordinates, μl. O_2 consumed; abscissae, time in minutes. The CO/O_2 suspension was kept in the dark from 0 to 15 min., illuminated with 548 mμ light from 15 to 30 min., and illuminated with white light from 30 to 45 min. *T*° C., 16·00; shaker speed, 96 c.p.m. *b*. Relative photochemical absorption spectrum of CO cytochrome oxidase in *Bacterium pasteurianum* (after Kubowitz & Haas, 1932); the arrow *A* shows the position of 548 mμ light and *B* the position of 420 mμ light. The former causes no photo-reversal of CO inhibition while the latter does.

DISCUSSION

These experiments, together with those of Gray (1928*a*, *b*, 1931), enable certain conclusions to be made about the causes of Senescence and the Dilution Effect in the spermatozoa of *E. esculentus*.

1. *Extracellular auto-intoxication.* No toxic or inhibitory substance diffuses out of the spermatozoa into the medium. Senescence is therefore not caused by extracellular auto-intoxication, nor is the Dilution Effect due to the dilution of an inhibitory substance in the medium.

2. *Exhaustion of intracellular substrate.* If, as has been shown, no toxic substances diffuse out of the spermatozoa, exhaustion of substrate is inadequate as an explanation of senescence, because an increase in dilution increases the total O_2 consumed, which would be impossible if substrates were not available. The Thunberg tube experiments confirm this theoretical argument. Unless it is postulated that the substrate which is oxidized by the appropriate dehydrogenase after motility and O_2 uptake have ceased is not oxidizable while the spermatozoa are motile, the conclusion is unavoidable that exhaustion of intracellular substrates is not responsible for senescence.

(3) *Intracellular auto-intoxication.* If (1) and (2) can be excluded, Gray's experiments (1928*a*, *b*) and those of Hayashi (1946), in which a linear relationship was found between total O_2 consumed and the square root of the duration of the experiment, would tend to confirm the intracellular auto-intoxication hypothesis. But against this, dilution of a sperm suspension in the middle of a manometric experiment causes an increase in O_2 uptake, both as regards rate and total O_2 consumed. It might be thought that the addition of sea water can only affect the concentration of spermatozoa, given that there is no inhibitory substance in the medium.

(4) *Allelostasis.* The comparative lack of O_2, and not lack of space to move, is responsible for sperm immotility in semen (Rothschild, 1949). When activated by O_2, spermatozoa in semen do not move so actively as after dilution and this is almost certainly due to their being so tightly packed together. There is, therefore, a case for dilution being an anallelostatic stimulus, though if this were the only explanation of the Dilution Effect, we should not expect concentrated and dilute sperm suspensions to have different physiological properties. Allelostasis itself implies auto-intoxication: if it did not, the total O_2 consumed per spermatozoon would be the same in dense and in dilute suspensions.

(5) *Exhaustion of link in metabolic cycle.* Once shed or artificially diluted, *E. esculentus* spermatozoa have a purely aerobic metabolism, being unable to glycolyse anaerobically, in the presence or absence of added substrate; glycolysis does not occur aerobically and there is no evidence that these spermatozoa contain any Pasteur enzyme as no acid production is observed in the presence of CO. It may therefore be that Senescence is partly due to the wearing out of some essential unit in the metabolic cycle, such as A.T.P.* (cf. mammalian spermatozoa, Mann, 1945*a*, *b*), and that these spermatozoa are unable to resynthesize this unit. Even in this case auto-intoxication must occur, as if this essential unit is available to account for the total O_2 uptake of a dilute suspension, why should it not be available for the consumption of the same amount of O_2 in a dense suspension?

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* No suggestion is made that the essential unit is A.T.P.

According to Barron, Seegmiller, Mendes & Narahara (1948), *Arbacia punctulata* sperm glycolyse anaerobically, but no experimental details are given in their paper.

(6) CO_2 *narcosis*. The experiments show that the depressed motility and O_2 uptake of concentrated suspensions are not due to the accumulation of CO_2.

There are several difficulties and confusing features in the arguments given above, and it is therefore necessary to consider whether the experiments suggest any different interpretation of Senescence and the Dilution Effect. The influence of supernatant sea water on the O_2 uptake of spermatozoa shows not only that dense suspensions produce no inhibitory substance, but also that the reverse effect takes place, i.e. there is a substance in the sea water obtained by centrifuging dense suspensions which prolongs the O_2 consumption of other spermatozoa suspended in this medium. There are good reasons for believing that this substance is derived from the spermatozoa, as the agitation of a suspension before centrifugation increases the stimulating action of the medium. Although it has not previously been suggested that the dilution of sea-urchin spermatozoa results in their losing some substance, Emmens & Swyer (1948) believe that the harmful effects of diluting rabbit spermatozoa are due to the diffusion of intracellular material from the sperm. Hayashi (1945, 1946) came to similar conclusions from examining the effect of seminal plasma on the viability and fertilizing capacity of *A. punctulata* sperm.

The experiments with different O_2 tensions and CO raise the possibility that there may be physiological differences between spermatozoa in dense and in dilute suspensions, although it is evident that O_2 diffusion is partly responsible for the observed differences. Winzler (1941) showed that the effect of low oxygen tension on yeast respiration varied considerably according to the concentration of yeast in the suspension, though no such variation was observed if O_2 uptake was measured with the dropping mercury electrode. He concluded that in order to prove manometrically that a biological system was sensitive to changes in O_2 tension, except at very low tensions, the results should be confirmed at different dilutions of the material, as well as at different rates of shaking; normally the latter is considered an adequate test that O_2 saturation is taking place (Dixon, 1943, p. 51). Winzler gives no explanation of this dilution phenomenon, which was observed in his manometric experiments, but not when the polarograph was used. The O_2 tension experiments recorded in this paper are in agreement with those of Winzler. Table 2 shows that O_2 saturation is apparently incomplete in dense suspensions at shaker speeds of 96 c.p.m. This raises the interesting possibility, which might be susceptible to experiment, that the lower rate of O_2 uptake per sperm observed in dense suspensions is apparent rather than real, a significant fraction of the spermatozoa being unable to respire through lack of O_2. A possible, though speculative and only partial, explanation of the phenomenon is that in dilute suspensions spermatozoa lose some substance to the medium, and that in these conditions interaction between oxygen and the sperm metabolic system is facilitated. The reduced inhibitory action of CO, even at a relatively low concentration (20% O_2 in CO), on dense suspensions, appears to fit in with this hypothesis, though it is also consistent with physical diffusion being a limiting factor; for the maximum inhibitory effect of CO will only be obtained when cytochrome oxidase is the limiting factor in the respiration. If diffusion is a limiting factor there will be less inhibition by CO, since the oxidase can be partially poisoned without the rate of O_2 uptake being affected. The effect of supernatant sea water in maintaining, but not increasing, the O_2 uptake of other spermatozoa suspended in it, may mean that there are physiological differences between spermatozoa in dense as compared with dilute suspensions. The experiments of Barron & Goldinger (1941) and Barron *et al.* (1948) on the effects of malonate, iodoacetate and halogenated alkyl amines on the O_2 uptake of *A. punctulata* spermatozoa could also be interpreted as confirming this view, as the stimulating action of these substances on O_2 uptake is quantitatively different according to the concentration of spermatozoa used in the experiment. Barron & Goldinger suggested that malonate and iodoacetate had no effect on the sperm themselves, but neutralized an inhibitory factor in seminal plasma. This explanation was not put forward by Barron *et al.* (1948) to explain the increased O_2 uptake in the presence of nitrogen mustards. In view of the observations of Gray (1928*a*, *b*) and Hayashi (1945, 1946) on the effect of seminal plasma on the motility, respiration and fertilizing capacity of sea-urchin spermatozoa, it seems unlikely that iodoacetate and malonate act in the way suggested.

The irreversible toxic action of high concentrations of CO has not previously been observed. The possibility that the CO/O_2 mixtures contain toxic impurities cannot be completely excluded; but it is quite possible that such high concentrations of carbon monoxide have an *indirect* and irreversible toxic action on the metabolism of the spermatozoa, as a result of the inactivation of cytochrome oxidase.

In mammalian spermatozoa, dilution is harmful from the point of view of fertilization (Chang, 1942): it may also be a relatively abnormal condition. In sea-urchins and many other marine organisms, in which fertilization is external, it would be difficult to assert that a short and active life is more or less useful than a longer and less active one, in achieving fertilization. Sperm dilution is of course inevitable when marine organisms spawn into the sea and the chances of there being sperm concentrations of 10^9/ml., except for very short periods of time, are remote.

No experiments have so far been done to test Gray's physical allelostasis theory. Although it might be thought that spermatozoa would have a lower rate of metabolism when closely packed, there exists the alternative possibility that the energy expended may be proportional to the work done against the constraining forces, in which case O_2 consumption would be higher in dense suspensions. Allelostasis is not a complete explanation of sperm immotility in semen, and the experiments described earlier indicate that this is also the case with regard to the Dilution Effect. It must therefore be concluded that there is no single factor responsible for Senescence, nor for the Dilution Effect. In the case of the former, intracellular auto-intoxication and perhaps the exhaustion of some essential link in the metabolic cycle are the most probable factors: in the case of the latter, the possible explanations are the loss of some substance from the sperm, oxygen deficiency and perhaps allelostasis at the same time.

A considerable decrease in O_2 uptake can take place without motility being correspondingly reduced. Robbie (1948) has come to the same conclusion from observing the action of cyanide on the respiration and motility of *A. punctulata* spermatozoa. O_2 uptake is therefore not an unequivocal index of motility. The significance of that part of sea-urchin sperm metabolism which is independent of movement is at present unknown.

SUMMARY

1. An investigation has been made into the causes of the interrelated phenomena of Senescence and the Dilution Effect in the spermatozoa of *Echinus esculentus*. The Dilution Effect is the increase in O_2 uptake and in movement observed when a concentrated sperm suspension, obtained by partial dilution of semen, is diluted with sea water.

2. The supernatant sea water obtained by centrifuging partially metabolized sperm suspensions contains no substances which depress the respiration or motility of the spermatozoa of the same species. This supernatant sea water has a small but definite protective action on spermatozoa in maintaining but not increasing their O_2 consumption. The effect is enhanced by agitating the suspensions before centrifugation.

3. *E. esculentus* spermatozoa do not glycolyse aerobically, anaerobically, or in the presence of 5% O_2 in CO. The absence of aerobic acid production proves that the reduced O_2 uptake per sperm of dense suspensions is not due to a lowering of the pH of the medium.

4. As the Dilution Effect is observed in manometric experiments in which CO_2 is continuously and adequately removed, neither accumulation of CO_2 nor a reduction in pH resulting from the accumulation of CO_2 are responsible for the reduced O_2 uptake of dense suspensions.

5. The O_2 uptake of dense sperm suspensions ($> 10^9$ sperm/ml.) is altered by changes in O_2 tension. O_2 consumption in 100% oxygen is nearly double that in 10% O_2 in N_2. An increase in O_2 tension has no stimulating action on dilute suspensions.

6. An atmosphere of 100% O_2 has a gradual toxic effect on dense and dilute sperm suspensions.

7. Carbon monoxide has a greater inhibitory action on dilute than on concentrated sperm suspensions.

8. The difference between the effects of varying O_2 tensions or CO on dense and dilute suspensions is partly, or perhaps wholly, due to gaseous diffusion being a limiting factor. This may partly explain the Dilution Effect.

9. Apart from photo-reversibly inhibiting respiration, CO exerts an irreversible toxic influence on sea-urchin spermatozoa.

10. Illumination of spermatozoa with a wave-length of light outside the region of photo-chemical absorption by CO cytochrome oxidase enables visual examination to be made in the presence of CO. The O_2 uptake of a suitable concentration of spermatozoa is 70% inhibited by 5% O_2 in CO. The same inhibition occurs when the

suspension is illuminated by light of wave-length 548 mμ, but there is no equivalent decrease in motility.

11. After motility and respiration have ceased sea-urchin spermatozoa still contain oxidizable substrates and the appropriate dehydrogenases as evidenced by their ability to reduce methylene blue in an evacuated Thunberg tube.

I am much obliged to the Director and Staff of the Scottish Marine Biological Station, Millport, where the experiments were carried out.

Since this paper was submitted for publication, Dr E. S. G. Barron has kindly informed me that after repeating the experiments referred to on p. 365 (Barron *et al.* 1948), on the glycolysis of sea-urchin spermatozoa, the views expressed in this paper, Summary, para. 3, were confirmed.

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STUDIES ON ANIMAL CAROTENOIDS

I. CAROTENOIDS OF THE BROWN TROUT (*SALMO TRUTTA* LINN.)

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(With Six Text-figures)

INTRODUCTION

This paper presents an analysis of the carotenoids of the brown trout (*Salmo trutta* Linn.), being the first of a series designed to throw light on the general functions of this class of pigments in animals. Apart from the role of the carotenoid derivative vitamin A as a component of visual purple, and the importance of some carotenes as vitamin A precursors, precise knowledge is almost entirely lacking of the part played by this group of substances in any metabolic process. Many of the lower Vertebrates, however, are known to accumulate large amounts of carotenoids and vitamin A in the liver and other tissues. The carotenoids are prominent also in the colour patterns of the skin, in which they are the characteristic pigments of the lipophore group of chromatophores. It seemed, therefore, that a quantitative study of their distribution in selected species of fish might prove a fruitful approach to clarifying the problems of their general metabolism. The brown trout was chosen for investigation since it is easily obtainable and possesses a characteristic colour pattern with at least two types of lipophores, the yellow or golden xanthophores and the red erythrophores. A further advantage of this species is that a considerable body of information already exists on various other aspects of its development and metabolism.

MATERIALS AND METHODS

Trout were obtained from a reservoir near Edinburgh, which was stocked annually with fish of the Loch Leven type from the hatcheries of the Howietoun and Northern Fisheries, Stirling. Natural spawning also occurred in a tributary stream, so that the fish population was in part self-replacing. Samples of fish were obtained by netting, which took place at intervals in connexion with another experiment. They were brought back to the laboratory alive and, as far as possible, all extractions of carotenoids were begun immediately after killing. A few fish were caught by rod-and-line angling and used immediately on return to the laboratory. When large amounts of material were being handled it was sometimes necessary to store certain portions, usually the first crude carotenoid extracts, and these were kept in the freezing compartment of a refrigerator. In general, all operations were carried out with a minimum delay and the temperature of all solutions kept as low as possible.

Separation and identification of pigments

The procedures used in these investigations for separating individual carotenoids were similar to those described by Zechmeister (1934), Zechmeister & Cholnoky (1941), Wald & Zussman (1938) and Baldwin & Beatty (1941). The pigments were extracted exhaustively from portions of minced tissues with acetone, methanol or petroleum ether (b.p. 40–60°), in some cases directly, but usually after grinding with sharp sand and anhydrous sodium sulphate. The combined extracts were transferred to petroleum ether by dilution with water, dried by shaking with anhydrous sodium sulphate, and then subjected to partition with 90% methanol. The petroleum ether was then evaporated under reduced pressure and the oily residue saponified with a few ml. of 6% KOH in ethyl alcohol for 1–2 hr. at 40–50° C. The saponification mixture was diluted with an equal volume of water and again extracted exhaustively with petroleum ether. This extract was washed successively with 5% KOH in 50% ethyl alcohol and water, and then partitioned once more between petroleum ether and 90% methanol. The saponification residue was acidified with a few drops of glacial acetic acid and extracted with further petroleum ether until no more pigment could be removed.

In this way the carotenoids were imperfectly separated into the following four fractions:

(1) Hydroxy-carotenoids, mainly xanthophylls, which were extracted from the tissues as free alcohols and were removed by 90% methanol at the first partition.

(2) Xanthophylls extracted from the tissues as esters, which were epiphasic before but hypophasic after saponification.

(3) Carotenes, which were epiphasic both before and after saponification.

(4) Acidic carotenoids, which remained as the potassium salt in the saponification residue after the carotenes and xanthophylls were removed by petroleum ether, and were only extracted therefrom after acidification.

These preliminary fractions were washed with water and dried with anhydrous sodium sulphate before being subjected to further fractioning and purification by chromatographic adsorption. The adsorbents most frequently used were alumina, (Savory and Moore's special preparation for chromatography) magnesium oxide (B.D.H. preparation low in arsenic) and finely powdered calcium carbonate. For rapid and clear development of the pigment bands much use was made of 2% methanol in petroleum ether, as recommended by Baldwin & Beatty (1941). The main pigment bands were in some cases separated by division of the column before elution. Usually, however, they were washed through the column by excess of the eluent and were collected separately. Pure petroleum ether, hexane, methanol, carbon disulphide and pyridine were all used as eluents at one time or another. It was usually necessary to adsorb and re-elute each fraction two or more times, often with different adsorbents and eluents, in order to obtain solutions showing constant spectroscopic properties and sharp absorption maxima. Imperfectly purified preparations often exhibited indefinite maxima when more than one pigment was present, or the absorption rose steeply in the violet region of the spectrum owing to the presence of non-carotenoid substances, probably mainly sterols, which absorb strongly in the near ultra-violet.

Absorption spectra were measured with a Hilger-Nutting Constant Deviation Wave-length Spectrophotometer (type D 186), which was calibrated at intervals by replacing the normal light source temporarily with a sodium or mercury vapour lamp and checking the wave-length scale with the sodium line at 589·0 mμ and with the mercury lines at 435·8, 491·6 and 546·1 mμ. The carotenoid solutions, brought to suitable concentrations in pure solvents, were measured in standard type Hilger cells of 1 cm. depth. Measurements were normally made in steps of 5·0 mμ, with a spectral band which was about 3 mμ wide at 475 mμ.

Solutions of standard preparations of *b*-carotene, lutein and astacene were used for comparison with unknown pigments, both in respect of their absorption spectra and their behaviour on chromatograph columns. The preparation and purity of these standards is discussed below in the section dealing with quantitative investigations.

THE CAROTENOIDS OF DIFFERENT TISSUES

Skin and fins

The pigments were extracted from chopped and ground portions of tissue, easily with acetone, pure petroleum ether or petroleum ether containing 2% methanol; slowly and with difficulty by pure methanol. They gave a clear golden solution in petroleum ether, which was entirely epiphasic before saponification. Crude extracts adsorbed on columns of magnesium oxide or alumina separated into two bands, a strongly adsorbed diffuse rose-coloured pigment at the top of the column and a more easily eluted orange-yellow pigment below. After saponification direct extraction of the diluted mixture with petroleum ether yielded a golden fraction, which exhibited the following properties when partitioned with methanol of different concentrations:

Partitioned between petroleum ether

and 70% methanol	All epiphasic
and 80% methanol	Mainly epiphasic
and 90% methanol	All hypophasic
and 95% methanol	All hypophasic

This behaviour suggested that the fraction contained dihydroxy-xanthophylls only. It was adsorbed on magnesium oxide and developed with 2% methanol in petroleum ether. Most of the pigment was contained in a strongly defined orange-yellow band, which was eluted easily with methanol or excess of the developer. In addition a small amount of the more strongly adsorbed rose pigment appeared at the top of the column, and in some experiments one or more faintly defined pale yellow bands below the main pigment. Addition of a solution of the lutein standard reinforced the main band, while *b*-carotene passed through the column without being adsorbed at all. The pale yellow trace bands were not isolated in sufficient amounts for further investigation. Their adsorption properties show that they were not carotenes. They probably were either monohydroxy-carotenoids or degradation products of carotenes.

The adsorption spectrum of the main orange-yellow band in hexane is compared in Fig. 1 with that of the lutein standard. The maxima at 475 and about 445 mμ agree well with the maxima of pure lutein in this solvent given by Morton (1942). In carbon disulphide the maxima lay at 512, 475 and about 450 mμ.

The saponification residue yielded after acidification a pigment which appeared to correspond with the rose-coloured fraction of the crude extracts. It was mainly hypophasic on partition between petroleum ether and 5% KOH in 50% ethyl alcohol, but returned to the petroleum layer on reacidification. It was purified by adsorption on magnesium oxide, from which it was eluted with difficulty by acidified methanol or pyridine. Its absorption spectrum showed a single broad band with the maximum at 475 mμ in hexane and about 500 mμ in pyridine. Crude preparations showed the maximum in pyridine between 490 and 495 mμ, but after adsorption and re-elution two or three times the maximum became stable at about 500 mμ.

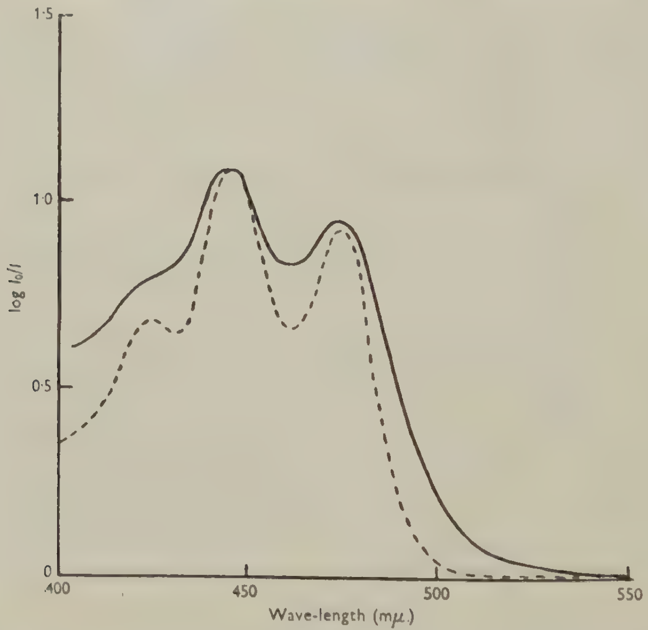

Fig. 1. Absorption spectra in hexane of the xanthophyll fraction of skin (continuous line) and of lutein (broken line). For ease of comparison the curves in this and in Figs. 2–5 have been adjusted to the same value of optical density at the main absorption maximum.

These properties serve to identify the second skin pigment as astacene. This was confirmed by comparing its absorption spectrum with that of that astacene standard (Fig. 2). A mixed chromatogram with the standard also gave a single intensified rose-coloured band on magnesium oxide. A few small purple crystals were obtained by partial evaporation of solutions in pyridine followed by dilution with a little water. These were insoluble in hexane, but dissolved readily in pyridine.

From their spectral properties one would expect lutein to be the pigment of the xanthophores and astacene of the erythrophores. To test this, pieces of skin were cut so as to exclude all red spots. On the chromatograph most of the pigment developed into the orange-yellow lutein band. Carefully dissected pieces of the red skin spots and the red tips of the adipose fins, on the other hand, developed intense rose-coloured astacene bands and relatively little lutein. Frozen sections were also cut of pieces of these tissues. Yellow skin apparently contained only xanthophores and melanophores fairly evenly distributed in the dermal layers, while the red spots and the tips of adipose fins showed dense aggregations of erythrophores, a few scattered melanophores, but apparently no xanthophores. These observations suggest that neither type of chromatophore contains a single pigment, but that the xanthophores contain mainly esters of lutein and the erythrophores mainly esters of astaxanthin.

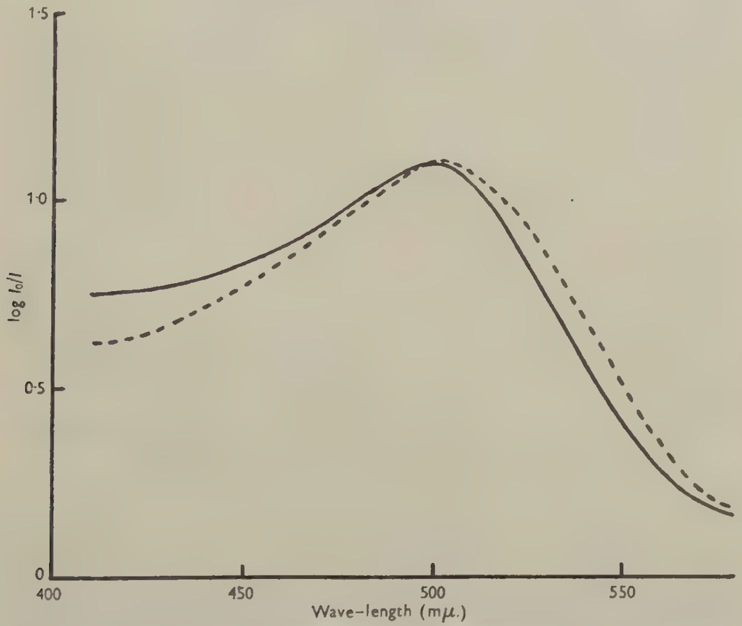

Fig. 2. Absorption spectra in pyridine of the acidic fraction of skin (continuous line) and of astacene prepared from lobsters (broken line).

Note. It is recognized that astacene or tetra-keto-*b*-carotene is usually regarded as a chemical artefact, being derived by oxidation from astaxanthin, which is the double *a*-ketol of *b*-carotene and is probably the naturally occurring pigment in living organisms. Most of our knowledge of astaxanthin, however, is derived from investigation of the properties of astacene, and in this paper the latter term is used.

Muscle

The main body muscles of many trout are highly pigmented, different fish showing all gradations from white, through various intensities of yellow and orange to a rich salmon pink. The amount of carotenoid extracted was roughly proportional to the intensity of pigmentation, none being obtained from fish with white muscle while well pigmented ones yielded a clear orange-pink solution in petroleum ether. Extracts of portions of pink muscle were analysed in the same way as described for skin. Some of the pigment was hypophasic between petroleum ether and 90% methanol before saponification. After saponification a further small fraction was removed by shaking the diluted mixture directly with petroleum ether, but the bulk remained in the saponification residue from which it was extracted after acidification. The acidic fraction possessed properties similar to those of the red skin pigment and the standard preparation of astacene, and after purification showed a single absorption maximum close to 500 mμ in pyridine (Fig. 3). The xanthophyll fraction when adsorbed on to magnesium oxide and developed with 2% methanol in petroleum ether separated into two closely associated bands: upper band, pink; lower band, orange-yellow. These moved down the column together and were separated with difficulty. The upper band was insufficient for further investigation, but the lower one, which contained most of the pigment, possessed the absorption maxima at 475 and 447 mμ in hexane characteristic of lutein (Fig. 4). Addition of lutein standard solution to the chromatograph increased the intensity of the lower band. It seems, therefore, that the hypophasic fraction of muscle contains two xanthophylls, principally lutein and probably a small proportion of its stereoisomer zeaxanthin.

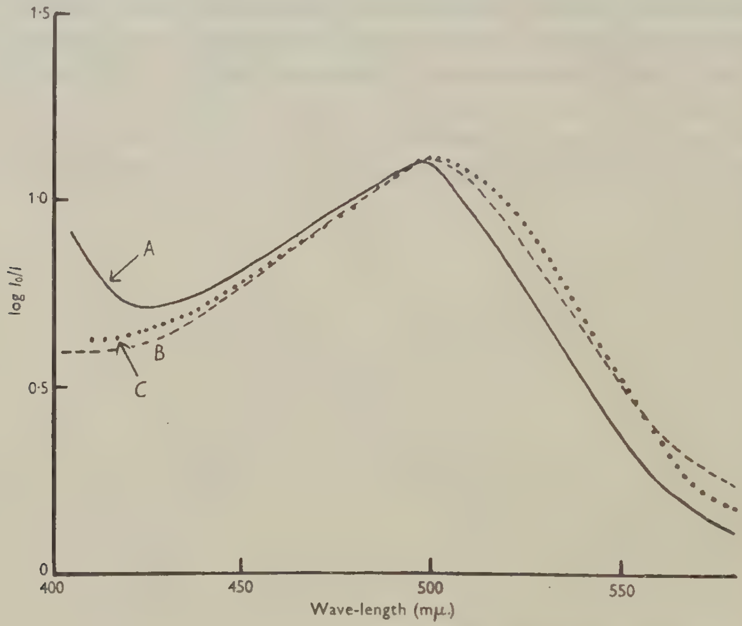

Fig. 3. Absorption spectra in pyridine of the acidic fractions of muscle (*A*), ovary (*B*) and astacene prepared from lobsters (*C*).

Some samples of muscle also yielded a minute fraction which remained epiphasic after saponification, and was therefore probably a carotene.

Frozen sections were cut at 20–25 μ of pieces of fresh muscle and others fixed in formal calcium (Baker). These appeared colourless, but on maceration or squeezing the fat which separated from the fibres had a pinkish tinge. Formal fixed sections at 10 μ, stained with Sudan IV and Sudan Black by the method of Baker (1945), showed numerous small globules of lipoid apparently lying between the muscle fibres.

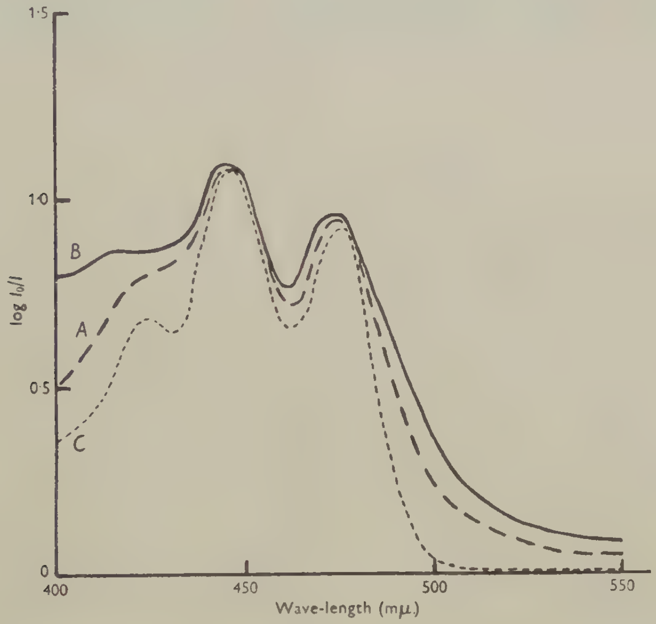

Fig. 4. Absorption spectra in hexane of the xanthophyll fractions of muscle (*A*), ovary (*B*) and of the standard lutein preparation (*C*).

Ovary

Extracts of the carotenoids of unripe ovaries behaved similarly to those of muscle. The pigment was nearly all hypophasic before saponification, indicating a preponderance of free hydroxy-carotenoids. After saponification the following three fractions were separated by the usual procedure: (1) an acidic fraction, which contained the bulk of the pigment; (2) a considerable xanthophyll fraction; (3) a relatively small hydrocarbon fraction. After further purification the acidic and xanthophyll fractions each yielded a single pigment similar to the astacene and lutein respectively of skin and muscle (Figs. 3 and 4). The hydrocarbon fraction when adsorbed on alumina and developed with 2% methanol in petroleum ether partially separated into two yellow bands, which moved down the column together and could not be completely dissociated from one another. This fraction gave absorption maxima at 453 and 485 mμ in hexane, and at 485 and 518 mμ in carbon disulphide, and therefore probably consisted mainly of *b*-carotene (Fig. 5), together with a small proportion of another carotene.

Liver

Liver carotenoids were more difficult to free from other lipoid substances than those of any other tissue, and in many experiments the absorption maxima were obscured by steeply rising absorption at wave-lengths shorter than about 450 mμ. To obtain sufficient pigment it was necessary to analyse mixed samples obtained by combining the livers of several fish. Although the relative proportions of the different fractions varied in different samples, in general rather more than half the pigment was hypophasic before saponification. When adsorbed on magnesium oxide or calcium carbonate and developed with 2% methanol in petroleum ether, the hypophasic fraction usually separated into the following bands: (1) one or more trace bands at the top of the column, not obtained in sufficient amounts for further investigation; (2) an orange-yellow band; (3) a golden yellow band. Bands 2 and 3 were closely associated and difficult to separate. Band 3 possessed absorption maxima at 448 and 478–479 mμ in hexane and at 475 and 510 mμ in carbon disulphide, which indicate lutein. Lutein standard solution added to the chromatograph intensified this band. Band 2 was only obtained in small quantities and separated

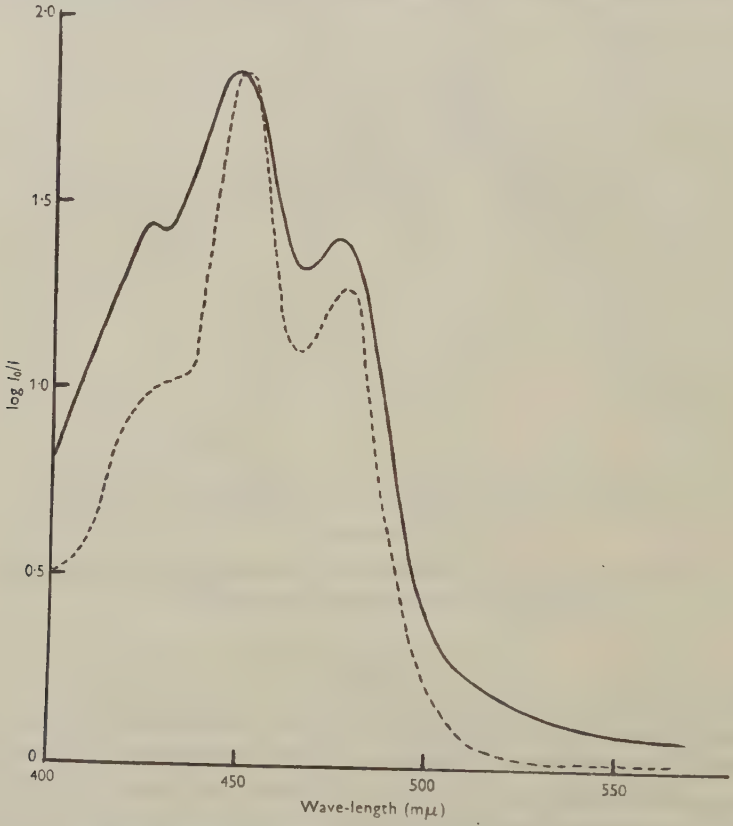

Fig. 5. Absorption spectra in hexane of the carotene fraction of liver (continuous line) and of *b*-carotene, B.D.H. (broken line)

imperfectly. It showed maxima at 454 mμ in hexane and at 485 and 517 mμ in carbon disulphide. The position of these maxima, and the fact that it lay above lutein on the column suggest that band 2 consisted of zeaxanthin. The trace bands at the top of the column were probably due to small amounts of xanthophylls with three or more hydroxyl groups.

The epiphasic fraction of the crude extracts yielded after saponification a further small hypophasic fraction, which could not be broken down further by adsorption. It showed indefinite maxima at 452–454, about 470 and at 485 mμ in hexane, and at 484–485, 510–511 and 520 mμ in carbon disulphide. These multiple absorption maxima suggest that both lutein and zeaxanthin were present, and possibly other xanthophylls also.

The hydrocarbon fraction was freed from xanthophylls by pouring on to a column of calcium carbonate, which retained the latter but allowed the carotenes to pass through. This fraction showed maxima at 425–426, 450 and 476 mμ in hexane (Fig. 5), and at 455, 483 and 505–510 mμ in carbon disulphide, and appeared to consist mainly of *b*-carotene, though the maxima at 476 mμ in hexane and at 505–510 mμ in carbon disulphide correspond more closely with the accepted values for *a*-carotene, as listed by Morton (1942). When adsorbed on alumina or magnesium oxide and developed with 2% methanol in petroleum ether, partial separation into two or even three bands was usually observed, but these remained too closely associated to be eluted individually. The livers therefore probably contained both *a*- and *b*-carotene and possibly other carotenoid hydrocarbons.

The saponification residues of large samples comprising the livers of twenty or more fish were tested repeatedly for an acidic fraction but only traces, too small for further investigation, were detected.

Liver contains therefore carotenes, free and esterified xanthophylls, but no astacene.

Eyes

Batches of about fifty whole eyes or enucleated posterior hemispheres were exposed to direct sunlight for a few minutes to bleach all visual purple. They were then ground with anhydrous sodium sulphate and extracted with acetone. The carotenoids were transferred to petroleum ether, in which they gave a yellow solution, by diluting the acetone to about 75% with water. The greenish yellow pigment which remained in the diluted acetone was assumed to be non-carotenoid. It fluoresced strongly green in ultra-violet illumination, and probably consisted of flavines. The carotenoid was all epiphasic before, but hypophasic after saponification. Only a single pigment, which corresponded closely with the xanthophyll of skin and muscle, was present. No carotene or astacene was detected in any experiment.

Other tissues

Other tissues examined, but which all yielded negligible amounts of carotenoids, were testes, brain, spleen, kidneys, stomach and intestine, the last two being washed clean of their contents. The testes, which are milky white, do not appear to contain carotenoids at any stage of the reproductive cycle.

QUANTITATIVE ESTIMATIONS

For measuring the amounts of the different carotenoid fractions of tissues a standard separation procedure was adopted involving only partitioning before and after saponification, and omitting chromatographic methods. Weighed portions of tissue were extracted exhaustively as described above, the combined extracts transferred to petroleum ether, which was then made up to a convenient measured volume, and the absorption due to the total carotenoids was measured. The petroleum ether was then evaporated under reduced pressure and the residue saponified in the usual manner. The diluted saponification mixture was extracted exhaustively with petroleum ether, then acidified and extracted once more until no further pigment could be removed. The combined extractions before acidification were separated into carotene and xanthophyll fractions by partitioning three times between petroleum ether and 90% methanol. The hypophasic fraction was returned to petroleum ether by dilution, and all three fractions were made up to convenient volumes for estimation in this solvent. This procedure was varied slightly to estimate the relative amounts of free and esterified xanthophylls extracted from any tissue. In these cases the free xanthophyll fraction was separated from the rest by partitioning three times with 90% methanol before saponification. The hypophasic fraction was then returned to petroleum ether and estimated separately.

It was appreciated that fractionation of carotenoids by partitioning alone is incomplete, since small amounts of other types can usually be detected on chromatograph columns even after repeated partitioning. It was considered preferable, however, to tolerate a small error from this source rather than to subject the fractions to be estimated quantitatively to further purification by chromatography, since the proportion of pigment recovered from the latter cannot be simply standardized. Moreover, since the absorption maxima of the carotenoids which are the subject of this investigation all lie close together at about 450 and 475 mμ in petroleum ether, it was not practicable to estimate their relative proportions in a single solution.

Estimations were made with a Unicam photoelectric colorimeter, type S21, using filters which transmitted only a single narrow wave-length band with maxima close to 450 and 475 mμ respectively. These were made up as follows: filter 450 mμ maximum, Ilford gelatine filters nos. 305, 803 and Q805; filter 475 mμ maximum, Ilford gelatine filters nos. 303, 803 and Q805. The gelatine strips were held between glass sheets cut from thin microscope slides, which were selected for optical clarity. The ultra-violet cut-out filter Q805 was included to reduce errors due to the presence of colourless impurities with strong absorption in the near ultra-violet, which were often apparent in the absorption spectra of liver and ovary extracts in particular. The inclusion of an infra-red absorbing filter, no. 803, was considered advisable since photocells of the type used in this instrument are highly sensitive in the near infra-red, and small amounts of substances absorbing in this region might cause serious errors.

Calibration curves were constructed from serial dilutions of petroleum ether solutions of standard preparations of carotene, lutein and astacene, and the concentrations of the appropriate fractions extracted from different tissues are expressed in terms of these standards. The standard preparations were obtained from the following sources:

b-carotene: B.D.H. pharmaceutical preparation. This proved in fact to be a mixture of carotenes, of which *b*-carotene was the most prominent. It contained also a small insoluble non-carotenoid residue.

Lutein: obtained from Prof. L. Zechmeister and prepared at the California Institute of Technology. Not guaranteed to be better than 90% pure. There was no insoluble residue.

Astacene: crystals prepared in the laboratory from fresh shells of lobsters. The preparation was free from other carotenoids, but contained a small proportion of impurity which was insoluble in organic solvents.

Probably none of the standards was in fact better than 90% pure, and the values obtained for the carotenoids of trout should be accepted with this reservation.

Table 1. *Carotenoid content of different organs of trout*

(All values expressed as γ/g. of fresh tissue. Estimated at 475 mμ maximum)

	Carotenes	Xanthophylls		Astacene	
		Free	Esters	Free	Esters
Whole skin and fins	0	0	87	0	81
Skin, excluding red spots	0	0	120	0	73
Skin, red spots	0	0	306	0	1608
Liver	7·5	8·5	3·5	0	0
Ovary	3	19	0	152	0
Muscle (well pigmented)	Trace	3·5	0	32·5	0
Eyes (posterior hemispheres)	0	0	0·2	0	0

The carotenoid contents of the various tissues investigated are summarized in Table 1. The figures presented are the average values of several determinations, except in the case of muscle. As mentioned above, white muscle yielded no carotenoid, and the figures given were those obtained from a highly pigmented specimen, which probably approximated to the maximum normally attained by this species. Yellow or orange-coloured muscle yielded rather lower values, 15–20 γ/g. for astacene and 1–2 γ/g. for xanthophyll. By far the largest concentrations of both pigments were found in the red skin spots and the red tips of the adipose fins. These were dissected out carefully, being separated as far as possible from the bony scales and underlying muscle, so that only small portions of the bright red dermis and epidermis were estimated.

It is of interest that the skins of fish of the Loch Leven type yielded the same concentrations of lutein and astacene as those of ordinary brown trout. Loch Leven trout are distinguishable by their silvery appearance, most marked on the sides and belly, compared with the golden or yellow colour of the ordinary race. This silvery surface appears to be a structural type of pigmentation in the scales or outer layers of the skin, since sections showed that the xanthophores, which lie mostly below the scales in the deeper dermal layers, are as numerous and as well pigmented in Loch Leven as in normal brown trout.

CAROTENOIDS IN RELATION TO DIET

It is well known that trout and other species of fish when kept in aquaria tend to lose the brilliant red and yellow pigmentation characteristic of the wild type. Xanthophores and erythrophores become few in number and those remaining contain little pigment. The fish can be kept indefinitely, will continue to grow and appear to suffer no other disability. If the loss of colour is due to lack of carotenoids in the usual aquarium diets, addition of specific pigments might be expected to show whether the fish are capable of converting one type of carotenoid into another; whether astacene, for example, is obtained direct from the food or is synthesized by the trout from one of the carotenes.

Yearling fish of about 8 cm. length and 5–9 g. were fed on horseflesh varied occasionally by chopped earthworms. The carotenoid content of this diet is negligible. After nine months on this régime very little red and yellow pigment was apparent. Microscopic examination revealed pale yellow xanthophores generally distributed throughout the skin and fins, and a few orange-pink erythrophores mainly at the tip of the adipose fins. Erythrophores had disappeared from the red spots along the lateral line. Estimations of the whole skin and fins of such fish showed that the total carotenoids, estimated as lutein, were reduced to about 10 % of the value normally found in wild fish (Table 2). Moreover, all the pigment remaining was lutein, whereas the skin of wild fish yielded lutein and astacene in about equal amounts.

Table 2. *Carotenoids of the skin and fins of experimental and wild trout*

(Carotenoid values measured at 475 $m\mu$ maximum; expressed as γ/g. fresh tissue)

	Total amount of carotenoid supplement fed to group (γ)	Duration of experiment (days)	Carotenoid content of all skin and fins (γ/g. tissue)		
			Lutein	Astacene	Total (estimated as lutein)
Wild trout	—	—	120	75	230
Depigmented trout (controls 9 months in aquarium on low carotenoid diet)	—	—	20	0	25
Trout reared in aquarium on 'natural' food	—	—	90	80	200
Depigmented trout fed on fresh ova of salmon	Astacene 20,000 Lutein 2,000	35	80	65	160
Depigmented trout fed with supplements of standard carotenoids:					
b-carotene	100,000	46	15	0	15
lutein	22,000	24	30	0	35
astacene	43,000	38	20	0	25

(*a*) Three groups, each consisting of three depigmented fish, were placed in separate tanks and their diet supplemented with the standard carotenoid preparations, one group receiving *b*-carotene, one lutein and one astacene. These were dissolved in pure arachis oil and injected into the body cavity of earthworms in which the septa between the segments had been destroyed by running a long needle through them. As much oil solution as possible was introduced, the wound closed with a clamp and the worms placed in the refrigerator for a few minutes until the oil was fairly hard and did not immediately flow out on removing the clamp. They were then fed to hungry fish, which habitually took them at once. No attempt was made to measure the exact amount of carotenoid each fish received. The total amounts given to each group and the duration of the experiments are recorded in Table 2. Allowing for differences in size and appetite, each fish probably received at least ten times its normal content of lutein and astacene and a comparable amount of *b*-carotene. No fish of any group, however, showed any increase of visible pigmentation, and the carotenoids of their skin and fins at the end of the experiment were as low as those of a control group which received no supplement. It seemed possible that the carotenoids, though ingested, were not absorbed in the gut of the trout. To test this several samples of faeces were collected and estimated, but these yielded only negligible amounts of pigment.

(*b*) Another group of eight depigmented yearling fish were fed about 5 g. of fresh salmon ova daily for 35 days. Their pigmentation increased visibly, and by the end of this period five had developed red flank spots and red adipose fins, and two of these even approached normal wild trout in brilliance. The background colour of their skin was rather more pink than in normal fish, and many of the xanthophores had a pinkish tinge. Four of this group were killed and the carotenoids of their skin and fins estimated. The astacene level was almost normal and the lutein about four times above that of the depigmented controls (Table 2). The remaining four were allowed to revert to the horseflesh diet. Their pigmentation faded once more and after three months none of them was distinguishable from the controls. Salmon ova contain lutein and astacene in about the same concentrations as trout ova. Each fish of this group received therefore about five times its normal content of lutein and about fifty times its normal content of astacene. The disproportion between the lutein and astacene content of salmon ova may well account for the abnormally pink colour developed by these fish, indicating that in the absence of sufficient lutein the xanthophores are able to lay down astacene esters.

(*c*) Another group of two fish was reared from ova in the laboratory and fed for over a year on 'natural' foods only. They were kept in a 'balance' tank with a permanent flora and fauna of Diatoms, filamentous green Algae of several species, *Elodea canadensis*, snails, Crustacea and insect larvae, to which rich infusions of Entomostraca, mainly *Daphnia*, *Simocephalus* and *Cyclops* species, and *Corethra* larvae were added from time to time. They fed exclusively on these, being given no horseflesh or earthworms. They grew well, attaining a size comparable with that of the control yearlings, and developed normal wild-type pigmentation. The carotenoid content of their skin and fins was found to be of the same order as in wild fish. Estimations of the carotenoids of the Entomostraca infusions leave little doubt that these provided the main source of the pigments during the first year of growth following the larval period. An infusion which consisted of about 85% *Daphnia longispina* (Müller), the rest being mainly *Cyclops* species, yielded 6520 γ of astacene, 1385 γ of xanthophylls and 120 γ of carotenes per g. dry weight.

ABSORPTION SPECTRA OF SINGLE CHROMATOPHORES

The absorption spectra of single xanthophores and erythrophores have been measured with a microspectrophotometer developed by Dr K. Pätau (late of the Kaiser Wilhelm Institut für Biologie, Berlin), details of which will be published elsewhere. Xanthophores show a sharp maximum at 487 mμ, a broad one in the region of 450 mμ and a small but definite one at 425 mμ (Fig. 6). The maximum at 425 mμ is interesting since the standard specimen of lutein in hexane shows one at the same wave-length, though it is absent or only just discernible in most spectra of tissue extracts. It is also of interest that though the maximum at 487 mμ is about 10–12 mμ towards the red from its position in hexane, the one near 450 mμ is less displaced and that at 425 mμ not at all.

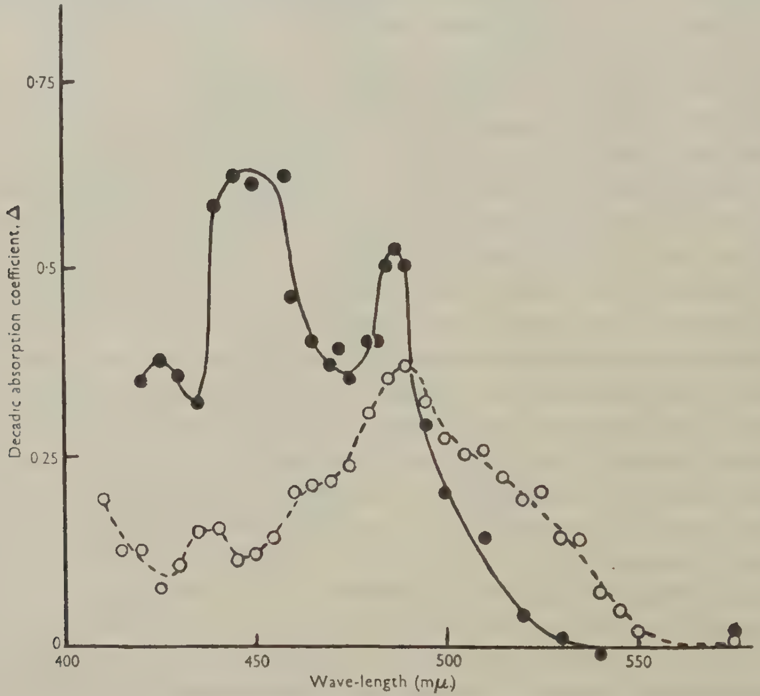

Fig. 6. Absorption spectra of a living xanthophore (continuous line and filled circles) and an erythrophore (broken line and open circles) measured with a microspectrophotometer.

The erythrophore shows a single main maximum close to 490 mμ (Fig. 6), and closely resembles the general shape of the spectrum of pure astacene. The small secondary maximum in the region 435–440 mμ is probably not due to a carotenoid.

THE CAROTENOID CONCENTRATION OF CHROMATOPHORES

A method described by Pantin (1923) for measuring pH inside the cell body of Amoeba was adapted for estimating the lutein and astacene concentrations of single chromatophores. Pantin compared the tint of neutral red indicator in the animal seen through the microscope with a series of standard buffered solutions containing the same indicator and placed so that they were reflected in the mirror of the microscope and focused by an achromatic condenser in the plane of the object. The two images were juxtaposed and compared directly. In the present experiments the optical density of single fully contracted xanthophores and erythrophores was compared with solutions containing known amounts of lutein and astacene respectively. Since it was desired to compare differences in optical density rather than differences of tint, it was possible to match the chromatophores by varying the thickness of the layer of standard solution. If it be assumed that the solutions conformed to Beer's Law, then the concentration of pigment in the cell should be proportional to the length of the column of standard solution required to match it; and knowing the concentration of the standard, the thickness and area of the chromatophore, the amount of pigment each cell contains can be calculated.

It was first necessary to prepare standard solutions the absorption spectra of which were as nearly as possible identical with those of the cell pigments. The spectra of carotenoids are affected by the solvent, the maxima being at shortest wave-lengths in hexane or petroleum, but displaced progressively towards the red end of the spectrum in solvents of higher refractive index. In carbon disulphide, the extreme case, the maxima lie about 30 mμ towards the red from their position in hexane. According to Wald (1943) carotenoid spectra in ordinary plant oils are displaced about 12 mμ, while in living tissues they are ordinarily about 15–20 mμ towards the red from their position in hexane. The standards were dissolved therefore in pure colourless castor oil. By adding carbon disulphide in various proportions to this solution it was possible to adjust the absorption maxima to any position within the desired range. These solutions, including that in pure castor oil, were used for matching the chromatophores.

The direct measurements of the absorption spectra of living chromatophores made with the microspectrophotometer developed by Dr K. Pätau indicate that the solutions in pure castor oil correspond best with the spectra of the living cells, and the data presented below are therefore those obtained by matching cells with the standards dissolved in this solvent without the addition of any carbon disulphide.

The solutions were sucked into lengths of capillary tubing, the bore of which was selected to give an image in the field of the microscope approximately the same size as the chromatophore. The tube was mounted horizontally and its position adjusted so that the solution was viewed accurately through the length of the column. Solution was then added or removed, care being taken to avoid introducing any air bubbles, until a match was obtained with the chromatophore. The length of the column was then measured to the nearest millimetre.

The chromatophores were examined in thin slices and frozen sections of fresh skin and fin tissues, mounted in frog Ringer's solution. They were first contracted by immersing for a few minutes in adrenalin 1/100,000 solution or in solutions containing a little barium chloride. When completely contracted the pigmented part of the cells appear more or less spherical. Their diameters, measured with a micrometer eyepiece, varied between 6·5 and 10μ. Well-rounded cells of 8μ diameter were used for matching, and it was assumed that they were spherical so that the maximum thickness of pigment through which the light passed was about the same as the cell diameter measured in the plane of the section. No allowance was made for scattered light, the possible lens effect of the cell, or the numerical aperture of the objectives used, since none of these factors could be evaluated simply. The microscope was focused just above the equator of the cell and the match made through the centre, i.e. through the maximum depth of pigment.

Table 3. *Estimation of the concentration of pigment in single chromatophores*

	Diameter of cell (μ)	Volume of cell (cu.μ)	Concentration of standard solution in castor oil (γ/ml.)	Absorption maxima of standard solution (mμ)	Length of column of standard required to match cell (mm.)		Concentration of pigment in cell (calculated) (γ/cell)	
					Min.	Max.	Min.	Max.
Xanthophore	8	380	166 (lutein)	485, 455	2	5	0·000011	0·000028
Erythrophore	8	380	90 (astacene)	486	68	115	0·00020	0·00034

The data obtained from matching xanthophores and erythrophores with standard solutions of lutein and astacene in castor oil are summarized in Table 3. Xanthophores were matched by columns from 2 to 5 mm. in length of a solution containing 166 γ/ml. of lutein. Assuming the pigment layer to be 8 μ thick, the calculated concentration within the cells was from 0·000011 to 0·000028 γ of lutein per xanthophore. Erythrophores were matched by lengths from 68 to 115 mm. of astacene solution containing 90 γ/ml., which correspond to values from 0·00020 to 0·00034 γ per cell. For both types the hue and saturation of the standards were very similar to the cells, which rendered subjective matching of their optical density relatively simple.

DISCUSSION

Previous investigators have established that carotenoids are distributed widely in fish, but few attempts have been made to identify specific pigments in definite types of cells, and quantitative information is very scanty. Carotenes, xanthophylls and carotenoid acids are all known to occur together or separately in different species. Lönnberg (1932, 1934, 1936), in a series of short papers, reported carotenes and xanthophylls in the skin of a large number of species. The methods he used did not, however, distinguish between free and esterified xanthophylls, so that the identification of carotenes is suspect (at least the carotenes could not be distinguished from xanthophyll esters). Lederer (1938) identified astacene in the skin of two marine and one fresh-water species. Among the Salmonidae, however, the pigments of muscle have received most attention. Emmerie, van Eekelen, Josephy & Wolff (1934) extracted a red lipochrome from the muscle of salmon (*Salmo salar*), which they named salmic acid, but its absorption spectrum and chemical properties show it to have been astacene. Sörensen & Stene (1938) identified the same pigment in the muscles of sea and fresh-water races of *Salmo trutta* as free unesterified astaxanthin, and obtained some quantitative data. Trout from Norwegian mountain lakes ('Gebirgsforelle') held about double the concentration found in fresh run sea trout. Bailey (1937) found astacene in the muscles of the North Pacific species *Oncorhynchus nerka* and *Salmo gairdneri*, and I have found it also in the char (*Salvelinus* sp.) of Scottish lochs (unpublished observations). It is obviously a widespread if not a general characteristic of the Salmonidae to store large amounts of this carotenoid in the muscles, probably as free astaxanthin.

Few investigators have paid much attention to carotenoids present in smaller concentrations, or to tissues other than muscle. In a little-known paper, however, Newbigin (1900) showed that the muscles and ova of Atlantic salmon contain both a pink and a yellow lipochrome, which were 'very similar to those of Crustacea and perhaps identical with them', and considered the pink one to be the same as crustaceorubin and tetroerythrin, which are the names under which astacene appears in the earlier literature. Examination of her data shows that the pink pigment almost certainly was astacene and the yellow, which was present in much smaller amounts, either lutein or a mixture of xanthophylls. She noted that the yellow pigment occurred also in the liver, though the methods she used would not of course distinguish between carotenes and xanthophylls.

My investigations on brown trout emphasize that the distribution of the different carotenoid types among the various tissues and even cells is highly specific. Lutein and astacene, but no carotene, occur in the skin entirely as esters. Their relative proportions in the yellow and red regions and the direct measurements of the absorption spectra of single cells show that they are the characteristic pigments of the xanthophores and erythrophores respectively. It seems that under natural conditions the two pigments do not occur together in the same cell to any great extent, although the experiments in which depigmented fish were fed on fresh salmon ova suggest that the xanthophores may take up astacene if the latter is available in great excess. Lutein and astacene occur also in the muscles and ova as free hydroxy-carotenoids. The liver contains both free and esterified xanthophylls, but no astacene. The largest concentrations of carotene, however, were found in the liver, though measurable amounts occur also in the ova and traces in the muscles. It seems likely that the carotenes, being the only group of these carotenoids known to act as precursors of the vitamins A, are mostly utilized in this way. Moreover, if the liver is the site of synthesis of vitamin A in fish, as it is believed to be in mammals, it is not surprising that most of the carotene occurs there.

Although the experiments in which depigmented fish were given supplements of pure carotenoids failed to show whether trout can convert or synthesize any of these substances, they support the view that the pigments of the lipophores, unlike those of the melanophores, are derived solely from the diet. According to McCay (1937) dried ova of salmon, goldfish, *Daphnia*, carrots and other foods rich in carotenoids have all been claimed to be effective in restoring or maintaining the wild-type coloration of trout kept in aquaria. Incidentally, goldfish (*Carassius auratus*) was one of the species in which Lederer (1938) identified astacene. Sumner & Fox

(1935) found that the xanthophylls of *Girella nigricans* were progressively reduced when the fish were kept in aquaria. After 5 months the carotenoid level was about 15% of normal, a reduction comparable with that found in my trout. Moreover, they found that supplements of carotene and xanthophyll added to the basal diet had no effect on the rate of depletion. The carotenoid supplements were added to the food by evaporation from petroleum ether in a vacuum desiccator, and are open to the same objection as in my experiments, namely that they may be altered in some way which renders them incapable of being absorbed or utilized. Sumner & Fox did obtain some evidence that their supplements passed through the alimentary canal of the fish and were voided with the faeces. It does not seem to matter from the point of view of utilization whether the pigments are ingested as esters or free hydroxy-carotenoids, as there is evidence that the former are acted upon by lipases in the alimentary canal and are absorbed as alcohols. Young & Fox (1936) found that the xanthophyll esters of shrimps fed to Pacific surf perches of the genus *Cymatogaster* could be recovered as free alcohols in the cells of the intestinal wall and the faeces. It is more probable that many of the pure carotenoid preparations are really chemical artefacts, as astacene is known to be a reaction product of astaxanthin. If this is so there is less cause for surprise that animals may fail to absorb the purified preparation although the corresponding naturally occurring pigment is utilized.

Other experiments, which will be described in a separate communication, support the view that trout cannot synthesize carotenoids, but utilize them efficiently if available in a suitable form. Xanthophores and erythrophores mostly develop during the latter part of the larval period, when the yolk sac is being rapidly absorbed, and if nearly all the carotenoids of the yolk are removed by operation soon after hatching the larvae develop very few and pale chromatophores.

Obviously the question of synthesis and utilization of carotenoids by fish requires careful investigation, and generalizations from experiments with one or two species would not be justified. Sumner & Fox (1933) considered there was presumptive evidence that *Fundulus* could convert carotenes to xanthophylls, and Lönnberg was struck by the fact that while xanthophylls were the predominating fraction in most fish, the Invertebrates on which they feed contained mostly carotenes. Speculations of this type are of little value unless based on quantitative information, for, as suggested above, fish may convert carotenes to vitamin A while progressively accumulating xanthophylls and other carotenoids. In *Girella* and the trout at least, two species which have now been searchingly investigated, there is no evidence that the fish can convert one type of carotenoid to another.

SUMMARY

1. The principal carotenoids of the trout are *b*-carotene, lutein and astacene. Carotenoids occur in the skin, muscles, ovary, liver and eyes, but the different types are distributed in a characteristic way in the various tissues. Carotene is found in significant amounts only in the liver and ovaries; lutein and astacene together in the skin, muscles and ovaries; lutein alone in the eyes. Xanthophylls, mainly lutein, occur also in the liver. Other carotenoids occur in trace amounts only.

2. The lutein and astacene of skin are in the form of esters. They are located in the xanthophores and erythrophores, of which they are the characteristic pigments. Yellow areas of skin yield mainly lutein, red areas mainly astacene, and their concentration in all parts of the skin is normally several times greater than in any other tissue. In muscle and ovaries both lutein and astacene are present as free hydroxy-carotenoids. Liver contains both free and esterified xanthophylls, the eye tissues only esters.

3. The concentration of the different carotenoid fractions in various tissues was measured with a photoelectric colorimeter in terms of standard preparations of pure carotenoids.

4. Direct measurements of the absorption spectra of xanthophores and erythrophores show that the former contain only lutein, the latter only astacene. Living xanthophores show absorption maxima at 487, about 450 and at 425 mμ; erythrophores a single maximum close to 490 mμ. The maxima in the blue-green are displaced some 10–12 mμ towards the red from their positions in hexane.

5. The concentration of pigment in fully contracted chromatophores was estimated by a visual matching method. Xanthophores were found to contain about 0·00001–0·00003γ of lutein per cell, erythrophores about 0·00020–0·00035γ per cell.

6. Trout kept on a diet low in carotenoids lose most of their red and yellow pigments. Astacene disappears from the skin, and the lutein falls to about one-sixth of the normal level. Feeding with natural foods rich in carotenoids may restore the skin coloration, and fish reared on a natural diet develop normal wild-type pigmentation. Supplements of pure carotenoids added to the basal diet failed to raise the pigment levels of depigmented fish. Trout do not appear to be able to convert one type of carotenoid to another.

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THE WATER-BALANCE PRINCIPLE OF CRUSTACEAN EYE-STALK EXTRACTS

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(*Received* 15 *August* 1948)

(With Four Text-figures)

INTRODUCTION

The pars nervosa of the pituitary gland is present in all classes of craniates and its active principles, viz. the antidiuretic, oxytocic and water-balance hormones, have been identified and estimated in mammals, birds, reptiles, amphibians, elasmobranchs and teleosts (Heller, 1941*a*, 1942, 1945). In *Amphioxus* the adenohypophysis is represented but apparently not the neurohypophysis (de Beer, 1926). A structure similar to the latter seems, however, to occur in ascidians (de Beer, 1926; Butcher, 1930). Homology of this structure, the subneural gland, with the vertebrate neurohypophysis has frequently been postulated on developmental grounds, first apparently by Julin (1881), and this conclusion is supported by the work of Butcher (1930) and of Bacq & Florkin (1935) who found that extracts of the subneural gland raised the blood pressure of the cat and stimulated the uterus of the guinea-pig. More recently Gray & Ford (1940) reported that extracts of crustacean eye-stalks produce an increase of water uptake in frogs similar to that observed after the injection of neurohypophysial extracts. There appears thus the possibility that posterior pituitary-like hormones occur in a class of invertebrates lacking in any structure homologous to the neurohypophysis. However, the similarity between the 'water-balance effect' of extracts of crustacean eye-stalks and that of extracts of vertebrate pituitaries is scarcely sufficient to characterize the eye-stalk factor as a posterior pituitary-like principle. It was therefore decided to investigate whether crustacean eye-stalk extracts showed any of the other actions of vertebrate neurohypophysial preparations.

MATERIALS AND METHODS

Eye-stalks of the brackish water-crab, *Carcinus moenas*, and of the fresh-water crayfish, *Potamobius fluviatilis*, were used.

Preparation of eye-stalk extracts. The method of extraction resembled closely that for mammalian posterior pituitary glands described in the *British Pharmacopoeia* (1932). Immediately after removal, the eye-stalks were dropped into acetone where they remained until the next day. The acetone was changed twice during the subsequent 24 hr. The material was then removed and dried in an evacuated desiccator over phosphorus pentoxide. The dry eye-stalks were stored at 5° C. either in sealed ampules or in a desiccator over phosphorus pentoxide. For the preparation of extracts a number of dry eye-stalks were powdered in an agate mortar, the powder weighed and washed into a hard glass boiling tube with 0·5 c.c. per eye-stalk of a solution containing 0·6% NaCl and 0·25% acetic acid. The extracts were boiled for 3 min., were then filtered and the residue washed twice with 0·5 c.c. of 0·6% NaCl solution. All extracts were assayed on the day of preparation. Control extracts from claw or leg tissue were prepared in an identical manner.

Assay of water-balance activity. English frogs (*Rana temporaria*) of an average weight of 20 g. were used. Several hours before the experiment started each frog was placed in a covered glass jar containing about 1 in. of tap water. Changes of weight of frogs kept under these conditions are small and inconsistent (Heller, 1941*b*). As a rule cross-tests were used to compare the effect of any preparations on the weight increase of a series of frogs. Usually five of a series of ten frogs were injected with one preparation, the other five with the other extract. After an interval of 48 hr. the grouping was reversed. Injections were made into a ventral lymph sac. The volume of each injection was kept uniform in any one experiment. Weighings were made 1 hr. before the injection, immediately before the injection, immediately after the injection and subsequently at hourly or half-hourly intervals. Before each weighing the frogs were carefully dried and the bladder thoroughly emptied.

Assay of oxytocic activity. Virgin guinea-pig uteri were used. The solution in which they were suspended was that recommended in the *British Pharmacopoeia* (1932), with the exception that magnesium chloride was omitted.

Assay of chloruretic activity. Adult male rats were used. The animals were starved for 18 hr. before the experiment started but water was allowed *ad lib.* They received 4% of their body weight of water by stomach tube, immediately followed by subcutaneous injection of the extracts which had been neutralized with NaOH and diluted to 2 c.c. The rats were then placed singly in glass metabolism cages fitted with urine/faeces separators. The urine was collected under paraffin. Chloride in the urine was determined by the method of Volhard (1878).

Assay of antidiuretic activity. Intravenous injections into unanaesthetized rabbits were employed (Heller, 1942).

Assay of pressor activity. Spinal cats were used.

Investigation of alkali-resistance of the oxytocic activity of eye-stalk extracts. Dudley's (1920) test was applied. It consists in mixing a volume of extract with an equal volume of 2 N-sodium hydroxide. The mixture is allowed to stand for 2 hr. at room temperature, and is then carefully neutralized with 2 N-hydrochloric acid and diluted for use.

Pituitrin and Pitocin (Parke, Davis and Co.) were used as the standard (mammalian) neurohypophysial preparations.

Statistical treatment of results. Fisher's 't' test was applied to estimations of the significance of differences of means. 'Small sample' methods were used for the calculation of 't' (Mainland, 1938). Allowance for the number of samples in any one series of experiments was made by determining the probability (P) from the tables of Fisher & Yates (1943). A probability of 5% was taken as the division between significance and non-significance, so that when $P < 0·05$, the difference between the values compared was regarded as significant.

RESULTS

Fig. 1 shows that extracts of eye-stalks of *Carcinus moenas* had a small but highly significant effect on the water uptake of normal frogs (t for 60 min. after injection = 5·26, $P < 0\cdot001$; t for 90 min. after injection = 3·89, $P < 0\cdot001$). Injections of the extract of ten eye-stalks of *Potamobius fluviatilis* per frog in a series of ten animals had a similar effect (results given as means with their standard errors, control experiments in brackets); percentage change in body weight 60 min. after injection = +3·9 ± 1·50 (+0·4 ± 0·21); 90 min. after injection = +3·1 ± 0·50 (−0·1 ± 0·39); 120 min. after injection = +2·0 ± 1·06 (−0·6 ± 0·35) and 180 min. after injection = +1·2 ± 1·06 (−0·9 ± 0·32); t for 60 min. after injection = 3·40, $P < 0\cdot02$, t for 90 min. after injection = 5·08, $P < 0\cdot001$. It is thus evident that a 'water-balance' principle occurs in the eye-stalks of both the brackish water *Carcinus* and the fresh-water *Potamobius*.

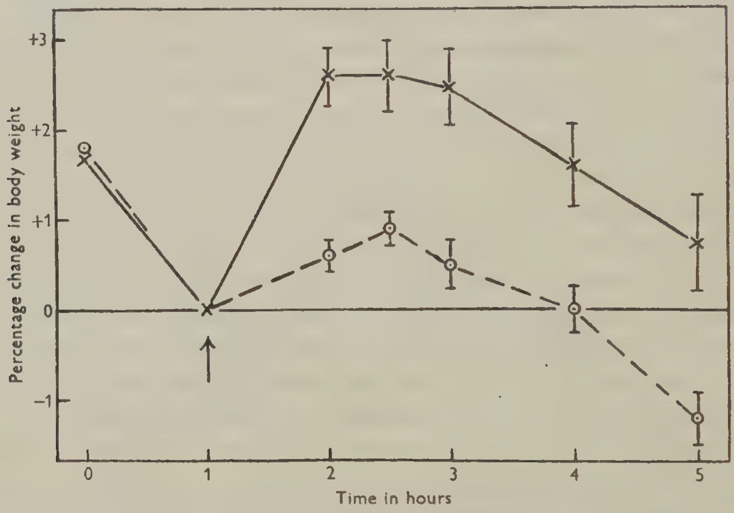

Fig. 1. The effect of eye-stalk extracts of *Carcinus moenas* on the water-balance of frogs immersed in water. ×——× mean percentage change in body weight of twenty-two frogs injected with the extract of ten eye-stalks each. ⊙- - -⊙ twenty-four frogs injected with control extracts. Control extract prepared from an amount of *Carcinus* leg tissue equal to the weight of ten eye-stalks, was administered per frog. Injections at the time marked by arrow. The vertical lines indicate the standard error.

Fig. 2 shows that the intravenous injection of an extract of ten eye-stalks of *Carcinus* had hardly any inhibitory effect on the water diuresis of an unanaesthetized rabbit, a result which was also obtained in five other animals. The small inhibitory effect noted in some of these experiments (see Fig. 2) was of the same magnitude as that produced by control extracts. It was, in any case, considerably smaller than the inhibition produced by 1 milli-unit of pituitrin. Two experiments with *Potamobius* eye-stalk extracts gave equally negative results. Experiments aimed at demonstrating a possible blood-pressure raising action of eye-stalk extracts in spinal cats were inconclusive since the eye-stalk extracts were found to contain traces of histamine-like impurities. However, it was sufficiently clear from the diuresis experiments that the crustacean eye-stalk extracts contained no factor comparable with the vertebrate's vasopressor-antidiuretic principle.

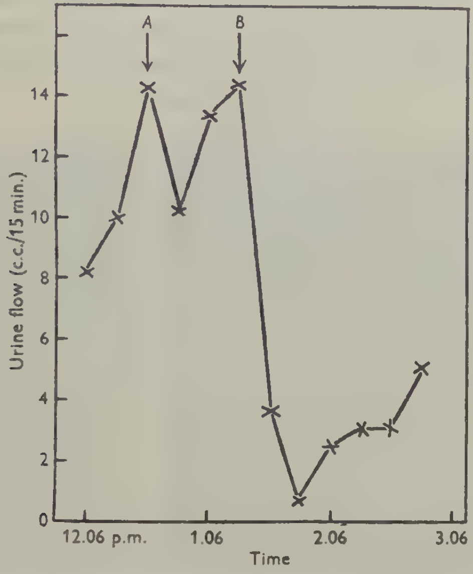

Fig. 2. Effect of intravenous injection of *Carcinus* eye-stalk extract on the water diuresis of an unanaesthetized rabbit. A = injection of an extract of ten eye-stalks. B = injection of 1 milli-unit of pituitrin.

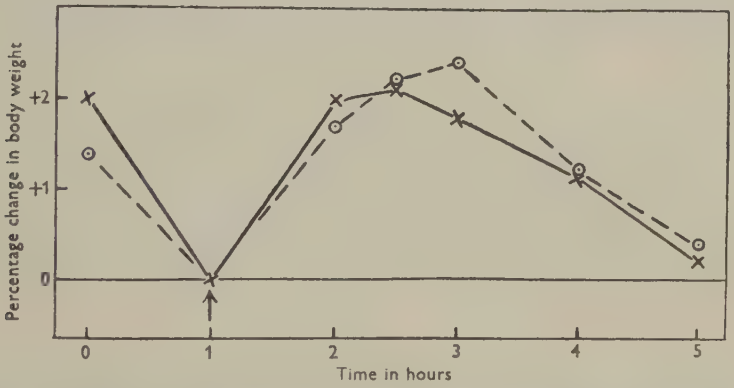

Fig. 3. The dose of pitocin required to equal the water-balance activity of an extract of ten *Carcinus* eye-stalks per frog. × —— × mean percentage change of body weight of fifteen frogs injected with the extract of ten eye-stalks each. ⊙– – –⊙ same frogs injected with 200 milli-units of pitocin per animal added to the appropriate amount of control extract. Injections at the time marked by arrow.

The amphibian water-balance effect of mammalian posterior pituitary extracts has been shown to be exerted by the oxytocic and not, as one would expect, by the vasoprenor-antidiuretic fraction (Heller, 1945). It was therefore of particular interest to test the eye-stalk extracts of oxytocic activity. Fig. 3 shows that the effect of the extract of one eye-stalk of *Carcinus* on the weight increase of frogs was approximately equal to that of 20 milli-units of pitocin. However, it will be clear from Fig. 4 that little or none of the oxytocic action of *Carcinus* eye-stalk extracts can be ascribed to a posterior pituitary-like principle. The extracts exerted an oxytocic activity equivalent to less than 2 milli-units pitocin per eye-stalk, but even this small effect was unspecific (control extracts of *Carcinus* leg tissue had much the same effect) and was not abolished by treatment with cold alkali (Dudley's test).

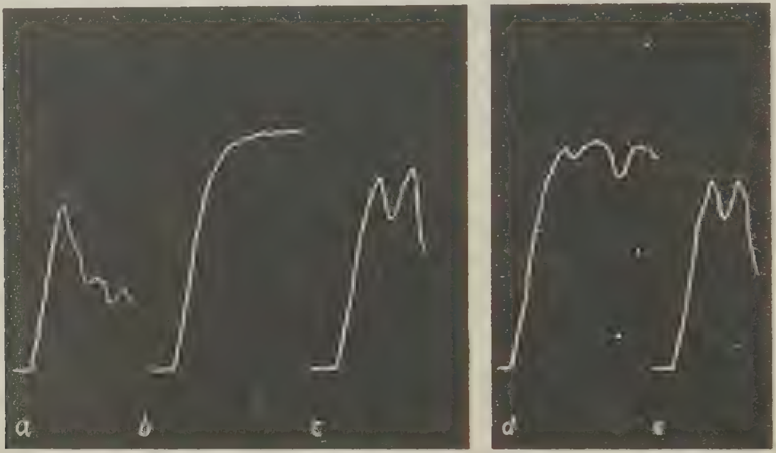

Fig. 4. The oxytocic activity of *Carcinus* eye-stalk extract. Isolated uterus of guinea-pig. *a* = effect of adding the extract of five eye-stalks which had been treated with cold alkali (= Dudley's test), *b* = 10 milli-units pitocin, *c* = extract of five eye-stalks, *d* = 10 milli-units pitocin, *e* = control extract of *Carcinus* leg tissue. The volume of fluid added to the bath was the same in each instance.

Moreover, extracts of eye-stalks of *Carcinus* or *Potamobius* failed to show the chloruretic action of the oxytocic fraction of mammalian posterior pituitary glands on rats (Kuschinsky & Bundschuh, 1939; Dicker & Heller, 1946). Six rats, for example, which had each been injected subcutaneously with an extract of ten *Carcinus* eye-stalks excreted a mean of 0·43 ± 0·130 mg. Cl/100 g. in 2 hr., six animals injected with control extracts excreted 0·75 ± 0·201 mg. Cl/100 g. in the same time.

DISCUSSION

The injection of extracts of eye-stalks of the brackish water-crab, *Carcinus moenas*, and of the fresh-water crayfish, *Potamobius fluviatilis*, was found to increase the body weight of frogs immersed in water. This effect resembles that produced by neurohypophysial extracts of vertebrates, which increase the uptake of water in amphibians (Heller, 1945). It seems likely that the increase in body weight of frogs injected with crustacean eye-stalk extracts was produced in the same manner: urine flow in such animals—though not accurately measured—was not noticeably inhibited and no specific vaso-active substances were found in the eye-stalk extracts.

The eye-stalk extracts had neither an antidiuretic action in rabbits nor an oxytocic action on the isolated guinea-pig uterus; they failed to exert a chloruretic effect in rats. There is thus no evidence that the crustacean and the vertebrate water-balance factors are homologous principles. However, the possibility that the water-balance factor of crustacean eye-stalk extracts is related to that in vertebrate pituitary extracts cannot be dismissed so easily. While the water-balance activity of mammalian neurohypophysial extracts follows closely their oxytocic potency (Heller, 1930; Oldham, 1936; Boyd & Brown, 1938) it has been shown (Heller, 1941) that this parallelism does not necessarily hold for non-mammalian pituitaries. For example, frog pituitary extracts which per gland exerted a water-balance effect equivalent to that of 800 milli-units of the standard (mammalian) posterior pituitary extract, contained less than 40 milli-units of oxytocic activity. These results suggest that the vertebrate water-balance factor may have to be differentiated not only from the antidiuretic but also from the oxytocic pituitary hormone. It is therefore conceivable that a substance occurs in crustacean eye-stalk extracts which is related to the water-balance principle of vertebrate pituitaries but unaccompanied by any of the other neurohypophysial activities.

Recent investigations (Heller, 1945; Jörgensen, Levi & Ussing, 1946) suggest an extrarenal regulatory action of the neurohypophysis on the water and mineral metabolism of lower vertebrates. The results of the present investigation would seem to raise the possibility that an eye-stalk hormone plays a similar role in crustaceans.

SUMMARY

1. Eye-stalks of the brackish water-crab, *Carcinus moenas*, and of the fresh-water crayfish, *Potamobius fluviatilis*, were extracted according to the pharmacopoeial method for the extraction of posterior pituitary glands.
2. Injection of such eye-stalk extracts resulted in a temporary increase in the body water of frogs. The increase produced by the extract of one eye-stalk of *Carcinus* was approximately equal to that produced by the injection of 20 milli-units pitocin (= oxytocic fraction of mammalian posterior pituitary extract).
3. The crustacean eye-stalk extracts had no significant oxytocic, chloruretic, or antidiuretic effect.

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THE EFFECT OF SEX-HORMONES ON THE PERFORMANCE OF A LEARNED RESPONSE

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(With Three Text-figures)

The restoration of copulatory responses in gonadectomized mammals following the administration of sex-hormones (Allen & Doisy, 1923; Marrian & Parkes, 1930; Boling & Blandau, 1939), and the return of cyclical variations in running activity, a characteristic of normal oestrous behaviour in the white rat, when an oestrogen is administered daily (Hemmingsen, 1933; Young & Fish, 1945), exemplify the fact that automatic adjustments of an animal's behaviour to its environment are governed by the co-ordinated action of its nervous and endocrine systems. In spite of the almost axiomatic quality of this major generalization, and of a considerable literature on the gross patterns of behaviour that are controlled by sex-hormones, few attempts have been made to analyse the character of the integration in specific instances. In view of the intricacy of the underlying processes, this is not surprising where learned responses are concerned. Differences that may be found in the rate or character of solution of a problem, after the administration of some drug or hormone, will merely show that some change in behaviour has taken place, without indicating what alteration in physiological function underlies the change.

The more immediate study of simpler acquired functional patterns of the central nervous system has, however, been eased by the use of the 'Goal-Gradient Alley' which Hull introduced in 1934 as an aid to the analysis of the dependent variables of maze learning. Hull's (1932) hypothesis of a 'goal-gradient' was derived from the observation that during maze learning rats run more rapidly in the later than in the earlier part of a maze, and that blind alleys nearer the goal tend to be dropped out of the response pattern earlier than those near the start. He therefore postulated an 'excitatory gradient' in motivation and strength of conditioning, the gradient increasing in intensity from the beginning of the maze to the food box. This excitatory gradient arose, he thought, from certain characteristics in the mechanism of learning. At each stage in the performance of a learned response, the strength of excitation is a product of the interaction between the sensory input at that particular moment of the response, and the neural pattern built up during the earlier stages of the response, and during all stages of previous similar responses. Hence there is a progressive increase in the strength of excitation as performance proceeds. The increase in strength results from the gradual addition of stimuli associated with the goal, and the elimination of stimuli that are not so associated.

The general validity of the main hypothesis and of its postulates, in given conditions, has been established. Hull (1934), arguing from the premise that the stronger an excitatory tendency, the more rapid will be the actions which result from it, showed that in a goal-gradient alley, which essentially is a long straight tunnel with a food box at one end, there is a speed-of-locomotion gradient paralleling the assumed goal excitatory gradient. Thus he demonstrated that in the process of learning to run the alley, rats ran faster near the end of the run, i.e. nearer the food box, than at the beginning. When learning was complete, the speed was the same in all sections of the alley. On the other hand, inhibition of the learned response by satiation or non-reward caused a return of the excitatory gradient—as manifested by an increase in the time taken by the rats to cover the first part of the alley.

Drew (1939), however, showed that a gradient in running speed, with higher speed nearer the goal, did not occur if training was restricted to a single daily run, but was present during learning only if rats were given successive trials during each day's training session. In his view the normal response lacked a gradient in running speed from the first to last section of the alley, and any improvement in performance during training was equally manifest in all sections. A gradient, he concluded, was a superimposition, and could occur in a learned response only in the presence of factors that tended to weaken and inhibit the connexion between stimulus and response at the moment of response. Increase in the gradient is therefore a measure of these factors, and may be found when trials are rapidly repeated, or following satiation or non-reward. When there is no inhibition, as for example, when one training run only is given a day, there is therefore no gradient. Drew restated these conclusions by suggesting that Hull's postulated excitatory gradient should be considered as a gradient of inhibition, which lessened as the goal was approached. Drew's conclusion finds support in the work of other experimenters (Buel, 1938; Buxton, 1940).

Miller & Miles (1935) had previously used the goal-gradient alley to analyse the effects of caffeine upon the speed-of-locomotion gradient caused by satiation and non-reward, and hence upon the strength of conditioning. Because there is available to-day a considerable body of knowledge on factors which may cause a change in performance in the goal-gradient alley, this technique seemed suitable for an analysis of the effects of sex-hormones on conditioning. It was accordingly used in the investigation described in the present paper.

There is little previous work with sex-hormones in this field of study. Herren & Haterius (1931) found that the central reflex time of the Achilles jerk is greater during oestrus than in dioestrus. Bilateral abdominal sympathectomy abolished this increase, which was therefore considered to be due to impulses arising from the hydration of the genital tract (Herren & Haterius, 1932). Later observations by Chauchard (1943) that the excitability of nervous tissue, as indicated chronaximetrically, is depressed by sex-hormones, is in accord with these earlier results. A still earlier observation of Kreps (1923) indicated that oestrus disturbs previously established conditioned responses in the dog, and more recently Vanderplank (1938) has shown that injections of oestriol will inhibit previously established conditioned reflexes in the rudd.

MATERIALS AND METHODS

The goal-gradient alley

The apparatus consists of a straight wooden alley, divided into five 3 ft. sections. Sections are interchangeable, and, when joined together, provide a 15 ft. enclosed tunnel 4 in. × 6 in. in cross-section, the roof of which can also be removed in sections. At each end of the alley is a short extra section, 1 ft. 3 in. in length. These serve as starting and reward boxes. The inner walls of the sections and end boxes are painted black, and the whole alley is mounted 3 ft. 6 in. from the floor on an iron frame.

The starting box is closed by a lid, and access to the first section of the alley is by a sliding door which is lifted vertically by hand. There is no lid to the reward box. The five sections and reward box are divided from each other by light, wooden, one-way swing doors. The opening of the sliding door at the start, and of the swing doors at the end of each section, are recorded electrically on a chronograph run from a constant speed motor, giving a paper speed of 1·1 cm./sec. The chronograph is remotely controlled, and is not housed in the same room as the alley.

Animals

Fifty-two sexually mature female white rats, weighing between 150 and 200 g. were used. They were received from a breeding station, and were reputed to be 3–6 months old. The litter relationships were not known. After spaying, they were housed in eight separate cages, containing 9, 8, 7, 7, 6, 6, 5, 4 animals respectively.

Methods of training

Training did not begin until at least a week after spaying, and after animals had fallen to 85% of their preoperative weight. The training technique is fairly simple.

On the first day animals are placed in the final section of the alley, with its roof off, and a feeding dish containing bread and milk is placed in the reward box. They are then gently pushed through the swing door between the last section and reward box, and allowed to feed for 30 sec. This is done three times consecutively, after which each animal is given 3 g. of bread in a separate cage. This procedure is repeated the second day, after which the animals are moved one section farther from the reward box each day, and the already learned section is roofed in. After the whole alley has been learned in this way, full training runs are given once every 12 hr., at 8.30 a.m. and 8.30 p.m. In this way complete learning, indicated by a small trial-to-trial variation in time for individual animals, is reached in 7–10 days.

The training method has been described in detail, because observation confirmed previous investigators' experience (Hull, 1934; Drew, 1939) that the form of response in the alley is affected by both the occurrence of inhibition and the type of training. Inhibition, as well as producing the typical speed-of-locomotion gradient described by Hull, also restores to a conditioned-response sequence the type of behaviour found earlier in training. Thus rats trained by being placed at the beginning of the alley and pushed to the end will, when inhibited, tend to run more slowly where they experienced most difficulty before (i.e. in the first section). Rats trained as in the present experiment will, on the other hand, usually run slowly in the last section.

In the present experiment the aim was to train each animal to a mean running speed near the population mean, and this criterion, rather than identical treatment for each animal, governed the exact number of training trials given to each animal. When learning was complete, each animal fed for 30 sec. in the reward box at every 12-hourly trial, and was then removed manually to a separate cage and given 6 g. of bread soaked in milk. Trial and error showed that this amount was always finished within 20 min., at the end of which time animals were returned to their home cages, where they were given water *ad lib.* If less food is given at each trial, motivation is so high as to mask any alterations in response that may be influenced by hormonal stimulation. The régime described, although providing a low motive, gives consistent results for individual animals, and a consistent population mean.

Experimental treatment

The fifty-two trained animals were divided arbitrarily into four equal groups, because statistical analysis showed that in so far as they did not differ significantly in either the means or the variances of their running-times they could all be regarded as forming a single homogeneous population.

Running time was then recorded after the injection of:

(1) Group I. 0·1 c.c. arachis oil only.

(2) Group II. 20γ oestradiol benzoate in 0·1 c.c. arachis oil.

(3) Group III. 20γ oestradiol benzoate in 0·1 c.c. arachis oil and 200γ testosterone propionate in 0·4 c.c. oil.

(4) Group IV. 20γ oestradiol benzoate in 0·1 c.c. arachis oil and 1 mg. testosterone propionate in 0·4 c.c. oil.

Before injection, the running times of the animals in each group were recorded twice at 12-hourly intervals. The results for the groups were once again analysed to discover whether there were significant differences either in population means or variances as between different times and different groups. None was found. Running-time was then measured every 12 hr. from the 6th to the 114th hr. after injection.

Methods of statistical analysis

The running times for the animals in each group were pooled, and the mean running-time and the variance of the groups were compared with control group I, which received an injection of oil only. Means were compared by Fisher's '*t*' test, and Fisher's method of analysis of variance, as described by Snedecor (1946), was used.

Differences were regarded as statistically significant when there was less than one chance in fifty that such a difference would attain the observed figure if the samples were random ones drawn from the same population.

RESULTS

The results obtained are presented in Figs. 1–3, and Tables 1–3.

Fig. 1 shows the mean running-time for each group plotted against the time in hours after injection. At each of the ten 12-hourly intervals of the experiment at which recordings were taken, the mean running-time of groups II (oestradiol alone) and III (oestradiol and 200γ testosterone) was greater than that of control group I. On the other hand, group IV (oestradiol and 1 mg. testosterone) was very similar to group I.

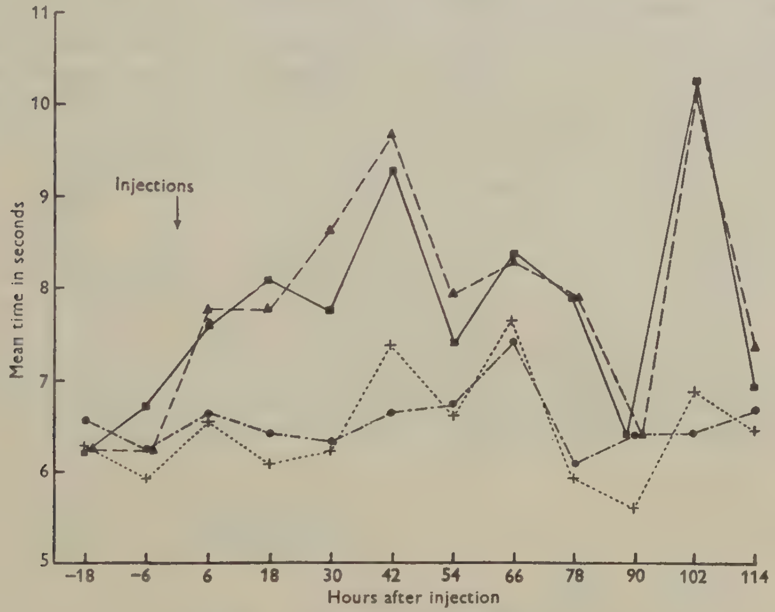

Fig. 1. Group-mean running-times

+------+ Group I, oil injection. ■——■ Group II, oestradiol benzoate 20γ.
▲——▲ Group III, oestradiol benzoate 20γ+testosterone propionate 200γ.
●—·—·—● Group IV, oestradiol benzoate 20γ+testosterone propionate 1 mg.

An analysis of variance (Table 1) of the group-mean running-time at each 12-hourly interval shows that the variance between the four groups after treatment was significantly greater than that within the groups.

Table 1. *Analysis of variance as between groups and within groups, of group-mean running-times for each 12-hourly session, from 6th to 114th hr. of experiment*

Source	Degree of freedom	Sum of squares	Mean square
Total	39	53·3	1·367
Variance between groups	3	28·0	9·33
Variance within groups	36	25·3	0·703

$F = 9{\cdot}33/0{\cdot}703 = 13{\cdot}27$. D.F. 3 and 36. $P < 0{\cdot}01$.

The differences between the group-mean running-times for groups I and II, and groups I and III are, however, statistically significant only at 78, and 18 and 78 hr. after injection respectively (Table 2). The mean differences at other times do not appear statistically significant because of the high variances of the individual observations.

Table 2. *Analysis of differences in group-mean running-times between groups I and II, and groups I and III, at 78, and 18 and 78 hr. after injection respectively*

Groups	Time after injection (hr.)	Difference (sec.)	S.E. difference	*n*	*t*	*P*
I and II	78	2·0	±0·56	13	3·570	<0·01
I and III	18	1·6	±0·61	13	2·623	<0·02
	78	1·6	±0·60	13	2·676	<0·02

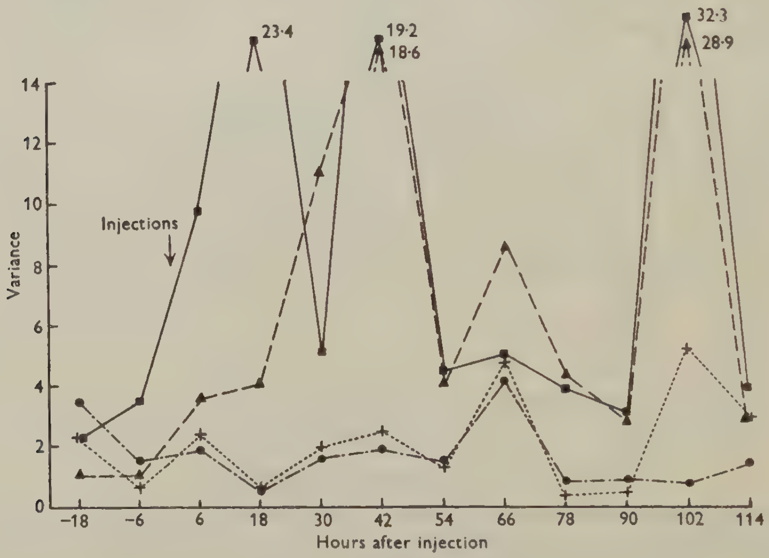

Fig. 2. Group variances.

+------+ Group I. ■——■ Group II. ▲———▲ Group III. ●—·—·—● Group IV.

Thus it will be seen from Fig. 2 that the variances of groups II and III are greater at every observed interval after injection than the variance of control group I. The variance of group IV, on the other hand, corresponds to that of group I. The records of each individual animal were analysed to discover why the variance was so high in groups II and III after injection, and hence why the mean differences at each 12-hourly interval were not always significant when compared to the mean running-time of the control group. It was found that although a significant behavioural change can be demonstrated in the majority of animals in groups II and III (ten out of thirteen in group II, and eleven out of thirteen in group III), this change did not take place at the same time after injection in each animal. Nor was the duration of the changed behaviour the same for all animals. Thus, whereas one animal exhibited a significant response decrement at one experimental session only, another continued to show it over three or four consecutive 12-hourly observations.

Table 3. *Analysis of differences in group-mean running-time for individual sections of alley, and in group-mean running-times for the whole alley, for the total experimental period, 6–114 hr. after injection*

Groups	Section of alley	Difference (sec.)	S.E. difference	*n*	*t*	*P*
I and II	1	0·6	±0·28	13	2·143	>0·05
	2	0·2	±0·10	13	2·000	>0·05
	3	0·1	±0·075	13	1·333	>0·20
	4	0·3	±0·145	13	2·069	>0·05
	5	0·3	±0·08	13	3·704	<0·01
	Whole alley	1·5	±0·41	13	3·653	<0·01
I and III	1	0·9	±0·26	13	3·460	<0·01
	2	0·3	±0·15	13	2·000	>0·05
	3	0·2	±0·15	13	1·333	>0·20
	4	0·2	±0·19	13	1·053	>0·30
	5	0·1	±0·23	13	0·435	>0·50
	Whole alley	1·7	±0·40	13	4·250	<0·001

Table 3 compares the mean differences in the average running-times of each group for the whole alley over the total experimental period. The times taken to run each section of the alley are also compared separately in this table and in Fig. 3.

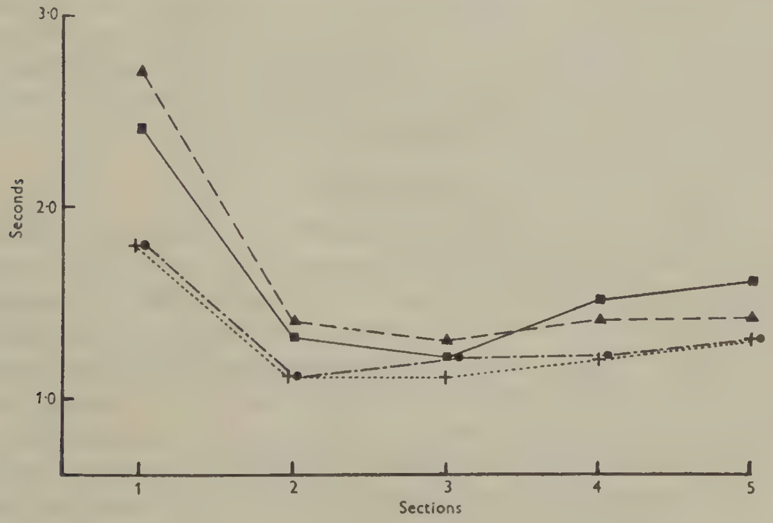

Fig. 3. Mean time taken in each section of alley during experimental period.
+------+ Group I. ■——■ Group II. ▲— — —▲ Group III. ●—·—·—● Group IV.

The differences in running-times for the whole alley between groups I and II, and between groups I and III, are highly significant.

Groups II and III also took longer than control group I in all sections of the alley. Group IV, however, behaved in the same way as the controls.

The difference in timing over sections was statistically significant in only the first section of the alley for group III, and the last section for group II. The difference approaches significance, however, for all sections except the third for group II, and the time spent by this group in the first two sections is significantly greater than that spent by control group I. ($n = 13$, diff. $= 0{\cdot}80$, S.E. diff. $= \pm 0{\cdot}29$, $t = 2{\cdot}818$, $P < 0{\cdot}02$.)

All these differences were not due to the different amount of oil given as a vehicle for the hormones, for from this point of view group I acted as a control for group II, and group III for group IV. It therefore follows that the administration of 20γ oestradiol benzoate to spayed mature female rats causes a response decrement of a characteristic type in a previously learned reaction. The same result is observed after the administration of 200γ testosterone propionate together with 20γ oestradiol benzoate. But if the dose of testosterone propionate is raised to 1 mg., the response decrement does not occur.

There were no differences between the behaviour of groups II and III. Nor were there any between groups I and IV. It is therefore probable that the response decrement found in group III was due to the oestradiol alone, and that 200γ testosterone propionate was too little, and 1 mg. testosterone sufficient to neutralize the effect of the injected oestrogen.

Vaginal smears were taken from all animals during the experimental period. No cornification was noted in any animal of group I. Marked cornification was first observed between 30 and 42 hr. after injection in all animals of groups II and III, and persisted long after the 114th hour. All animals of group IV also showed some cornification. But although cornified cells appeared 30–42 hr. after injection, their number was much less than in groups II and III, and they disappeared 24–96 hr. after their first manifestation. This is further evidence that the 1 mg. of testosterone propionate neutralized the injected oestrogen.

DISCUSSION

These experiments show that oestrogens can cause a decrease in the speed of performance of a learned response. The increase in time taken to run the alley is due mainly to an increase in the time taken to traverse the first two and the last sections of the alley.* This type of decrement in response has been observed by other investigators (Hull, 1934; Drew, 1939) and confirms the previously established principle that weakening of a learned response restores to that response behaviour which occurs in earlier stages of training.

The possible physiological mechanisms underlying the gradual weakening of a learned response may be considered seriatim. Loss of a conditioned response may result from the repeated rapid elicitation of the response (adaptation), from alteration in the strength of the conditioned stimulus—for example by satiation or repeated omission of a reward for performance (internal inhibition), or from interference due to the interaction of other stimuli during the elicitation of the response (external inhibition) (Pavlov, 1927; Hilgard & Marquis, 1940).

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* Corresponding results were obtained in an earlier and unreported set of experiments carried out in 1939–40 by two of us (J.W.B.D. and S.Z.).

That the response decrement did not represent adaptation in the sense in which the term is defined is indicated first by the fact that the conditioned responses showed little tendency to diminish with the passage of time, and secondly by the fact that the present experiment did not allow rapidly repeated elicitation of the response.

It also seems clear that the decrement in response was not due to internal inhibition, since the strength of the conditioned stimulus (hunger) was kept constant. After being run, all rats consumed the 6 g. of food they were given in a separate cage. Although motivation was never maximal, there is no indication of its alteration. Lack of a significant alteration in the mean body weight of each experimental group between the 6th and 114th hr. of the experiment supports this conclusion.

The explanation for the weakening of response presumably falls, therefore, into the category of external inhibition. This may be either from an alteration in the quality or in the quantity of the extraneous stimuli acting upon the organism during performance, or a direct effect of oestrogens upon the functional equilibrium of the central nervous system. Both may in fact be facets of the same process.

It is known that the water content of a wide variety of tissues is affected by oestrogenic stimulation (Zuckerman, Palmer & Bourne, 1939). That of the skin, uterus and vagina increases 6–18 hr. after injection of $0{\cdot}1\,\gamma$ oestradiol, and decreases at 24 hr. There is a further rise in water content from 30 to 60 hr., after which water is no longer retained in the tissues, and the water content drops to below the normal resting level. The changes in water balance in the brain, heart and gut are to a certain extent reciprocal to those observed in the uterus. There is a rise in water content 24 hr. after injection of $0{\cdot}1\,\gamma$ oestradiol, followed by a fall at 30 hr., and a further rise between 48 and 66 hr., when the uterus is beginning to lose water.

That the uterus, which is but one of the tissues involved in these widespread changes in water balance (Astwood, 1938), may be a source of inhibitory impulses to the central nervous system has been suggested by Herren & Haterius (1931, 1932). It is also known, however, that the cyclical alterations in spontaneous activity that characterize the oestrous cycle of rats do not depend on impulses arising from the genital tract (Durrant, 1931) and that they therefore depend upon changes in other tissues. Direct alteration in the functional equilibrium of the central nervous system after oestrogenization is shown not only by the changes in water balance, but also by the alteration in threshold to some environmental patterns of stimulation (for example, sexual patterns); by the selective facilitation of certain motor responses relating to these patterns; by the alteration in certain autonomic reactions; and by changes in equilibrium within the autonomic nervous system. Hydration of the brain, which may cause a relative cerebral anoxia, might in itself lead to alterations in the function of the central nervous system, and hence in its ability to subserve the performance of a learned response. For example, Welsh (1943) has shown that the responsiveness of the cortex to stimulation, and the vigour of its spontaneous activity, decrease markedly under conditions of anoxia.

There is considerable evidence, however, of the specific effect of gonadal hormones on autonomic equilibrium. Anderson (1940*a*) has shown that female rats are less timid when sexually receptive, and that timidity increases after spaying (Anderson, 1940*b*). It has also been shown that the timidity of spayed rats decreases after oestrogenization (Anderson & Anderson, 1940). Heat periods can recur in the absence of afferent impulses from the genitalia (Sherrington, 1906; Bacq, 1932; Bard, 1935), and the results of experiments involving trauma to the central nervous system have led many workers to the conclusion that the motor patterns of female copulatory behaviour must result from facilitation by oestrogens of impulses from autonomic motor centres located between the midbrain and anterior hypothalamus (e.g. Brooks, 1937; Bard, 1940; Brookhart, Dey & Ranson, 1941). A specific affinity of sex hormones for cells in this region has therefore been postulated. Effkemann & Strüver (1939) have further shown that oestrogens have a sympatheticotropic action, which they measured by alterations in pupillary diameters. Effkemann (1939) also showed that testosterone had a parasympatheticotropic action when measured by the same method, and thought that his results were also due to the specific action of gonadal hormones on diencephalic autonomic centres.

External inhibition may therefore result directly from impulses arising from tissues which retain water after oestrogenic stimulation, or indirectly from the effect of this hydration upon other functions of affected tissues (for example, the effect of hydration upon the blood supply to the brain), or indirectly from the action of oestrogens upon autonomic adjustment reactions. The importance of the last possibility lies in the fact that the strength of any superimposed and acquired adjustment involving learning must depend upon the integrity of the autonomic adjustment an animal makes to its environment. Alteration in autonomic adjustment may follow variation in the quality of sensory input, and such alteration may therefore be the antecedent to the presence of external inhibition to a learned response.

SUMMARY

1. The Hull 'Goal-Gradient Alley' is described, and its advantages in the analysis of the effect of hormonal stimulation upon conditioning are discussed.

2. The rate at which spayed rats run the goal-gradient alley was tested before and after the administration of oestrogen alone and oestrogen combined with androgen.

3. It was found that a decrement in the speed of running for food occurs at varying times from 6 to 102 hr. after the administration of oestradiol benzoate.

4. The decrement is still apparent when testosterone propionate and oestradiol benzoate are administered in the proportion 20:1, but is not present when the proportion is 50:1.

5. The possible physiological mechanisms of this response decrement are considered.

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THE PHYSIOLOGY OF CONTRACTILE VACUOLES

V. THE EFFECTS OF SHORT-TERM VARIATIONS OF TEMPERATURE ON A FRESH-WATER PERITRICH CILIATE

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(With Five Text-figures)

INTRODUCTION

It has long been known that increase of temperature, within reasonable limits, causes an increase in the frequency of pulsation of the contractile vacuoles of various Protozoa. (For references see the review by Kitching, 1938*b*, and the recent paper by Smyth, 1942.) Unfortunately, earlier workers did not follow the changes in maximal diameter of the contractile vacuole which may result from changes in temperature, so that no information has hitherto been available as to the relation between temperature and rate of vacuolar output.

In many Protozoa, and especially in fresh-water forms, there is a considerable exchange of water between the body and the outside. The contractile vacuole bales out water continually, and there must be a corresponding uptake into the body from the external medium. For the purpose of the present investigation it is necessary to consider more closely the various components of this exchange, since the effects of temperature on vacuolar output can only be understood in relation to the water balance as a whole.

Uptake of water from the external medium takes place in part by means of food vacuoles, but these account only for a minor portion of the output in those fresh-water Protozoa in which this question has been investigated (Kitching, 1938*b*, p. 413, and 1939). The greater part of the uptake must take place either through the general body surface or through some restricted part of this. There is as yet no satisfactory evidence by means of which a choice can be made between these two alternatives. Eisenberg (1925), Frisch (1937), and Mast (1947) have concluded that uptake is restricted to the 'oesophageal sac' at the base of the pharynx. The evidence rests on the observations that neutral red staining first appears in this neighbourhood (Frisch) and that food particles become concentrated in the oesophageal sac shortly before a food vacuole is pinched off (Mast); it does not seem sufficient to justify the claim that water is exclusively or even predominantly taken up through the oesophageal membrane. Accordingly the other hypothesis, being the simpler, will be utilized here for purposes of discussion; the conclusions drawn in this paper are not affected by this choice. In any case the uptake of water is presumably due to osmosis; it is difficult otherwise to explain the susceptibility of vacuolar output to changes in the osmotic concentration of the external medium (Kitching, 1938*a*).

Loss of water from the body to the external medium takes place through the contractile vacuole; this probably is the chief means in most fresh-water Protozoa. There is also some loss of water by extrusion of food vacuoles at the close of their digestive cycles. In *Vorticella*, according to Mast & Bowen (1944), the amount of water lost in spent food vacuoles does not fall far short of that which was taken up in these vacuoles when they were first formed; and the same may be true of *Paramecium* (Mast, 1947). In these cases the rate of output of the contractile vacuoles must be approximately equal to the rate of osmotic uptake of water into the body from the external medium. It is difficult to observe the elimination of spent food vacuoles in the smaller peritrich ciliates, but I am very doubtful whether the amount of water lost in this way is as great as that taken up in food vacuoles. However, the rate of uptake of water in food vacuoles is in any case fairly low (see p. 406), so that here also the rate of output of the contractile vacuole cannot differ greatly from the rate of osmotic uptake.

Maintenance of the normal body volume demands that the rates of uptake and of loss of water remain equal, though not necessarily constant; any change in one must be met by a compensatory change in the other. The present investigation, therefore, deals with the effects of temperature on the rate of vacuolar output and on the body volume of certain peritrich ciliates. From these the effect of temperature on the rate of uptake of water from the external medium can be inferred. This rate cannot easily be measured directly, but additional evidence relating to it is also presented in the following paper.

METHODS

Peritrich ciliates have been used throughout this series of investigations both because of their regular body shape and because it is much easier to work with a sessile organism. *Carchesium aselli* (previously referred to incorrectly as *Zoothamnium* sp. by Kitching, 1938*a*) was used for the experiments described in this paper. It is commonly found on the hairs on the legs of the fresh-water isopod *Asellus aquaticus*. The *Asellus* were collected from a pond near Bristol, and were kept for 2 weeks or longer in a large aquarium tank containing pond water at pH 7·7–8·0 and 12–15° C. and well supplied with organic detritus.

In a number of preliminary experiments a leg of *Asellus* was pulled off and mounted in a hanging drop of pond water under a cover-glass sealed with vaseline over a small glass cell. The cell and cover-glass were surrounded by well-stirred water at controlled temperature in a water-bath mounted on the microscope stage. The objective was immersed, and for low temperatures dry air was blown through the microscope to prevent condensation on the lenses. This method gave good control of the temperature down to freezing-point, but was found unsatisfactory for general use because in the majority of cases the rate of output of the contractile vacuole fell off in control experiments at constant temperature (15° C.). It was therefore abandoned except for experiments at the lowest temperatures.

Accordingly I reverted to the method used in previous papers of this series (Kitching, 1934, fig. 1), by which the organism is irrigated throughout the experiment with a solution at controlled temperature. The solution used for irrigation was allowed to drip at the edge of the cover-slip from a glass 'supply tube', and was led away at the other side of the cover-slip by means of filter-paper to a funnel. The supply tube passed through a water jacket the temperature of which could be altered quickly, and the actual temperature under the cover-glass was measured by means of a thermocouple junction placed within 2 mm. of the organism. Pond water at pH 7·7–8·0 was used. In each experiment the organism was irrigated under experimental conditions at 15 ± 0·5° C. for 30 min. before observations were begun, and this preliminary period is not included in the durations of treatment given in Table 1.

The maximal diameter of the contractile vacuole (attained just before systole) was measured with a screw micrometer eyepiece. The time of systole was recorded to the nearest second. From this information it is possible to estimate the rate of output for each cycle of the vacuole observed, and this has been done for purposes of illustration in Fig. 2. However, since (except in certain specified cases) there was no evidence to suggest other than random fluctuations in ultimate diameter (as measured) and in the interval between systoles, the mean rate of output for each temperature was calculated from the mean ultimate diameter and the mean interval. The range of variation found in typical experiments is illustrated in Fig. 2; the significance of the readings is so apparent as to render further statistical treatment superfluous.

In some experiments observations were also made of the approximate size of the food vacuoles and of their time of uptake. The exact size of food vacuoles is difficult to determine as these are not spherical when first taken up, and no significant changes in size are claimed in this paper. The significance of the changes observed in the interval between the times of uptake of successive food vacuoles can be judged from the means and standard errors given in Table 2.

In view of the importance of knowing how much food vacuoles contribute to the general water balance, a more precise estimation of the upper limit of this contribution was attempted in a few cases at 15° C. Food vacuoles originate in a spindle-shaped depression, known as the oesophageal sac, at the base of the pharynx. This sac is periodically pinched off as a food vacuole and is reformed. The volume of the oesophageal sac, at its maximum, was estimated from the length and breadth on the assumption that the shape was that of a sphere capped by two tangential cones. Figures obtained in this way give an upper limit for the uptake of water by means of food vacuoles.

Observations were also made on the activity of the adoral cilia which encircle the disk at the free end of the organism. In some experiments these stopped beating, and in some they underwent periodic intermissions.

An indication of possible changes in the body volume of the organisms under experiment was obtained from measurements of the length and breadth. The length as measured in this work usually did not include the disk, as the constriction or neck immediately below this provided a more clearly defined line from which to measure. The breadth was, of course, measured at its greatest. The range of variation in estimates of the length and breadth under constant environmental conditions did not normally exceed 1 μ in either measurement, so long as the organism remained extended in the plane of focus.

In a few experiments organisms were subjected to a series of temperatures, starting with 15° C. In most cases, however, for reasons explained later, the organism was observed (*a*) at 15 ± 0·5° C., (*b*) at a selected experimental temperature ± 0·5° C., (*c*) at 15 ± 0·5° C.

RESULTS

Control experiments

During irrigation at 15° C. the vacuolar output was maintained at an approximately steady level. This was shown in the initial period of all experiments, and throughout control experiments lasting up to 2 hr. The limited scatter recorded during the initial period of the experiment illustrated in Fig. 2 is typical. Food vacuoles were also taken up at regular intervals, the cilia beat actively, and the size of the body remained constant.

The contractile vacuole

A few experiments were carried out in which the organisms were subjected to various temperatures in succession. Results are given in Fig. 1. It will be seen that the rate of vacuolar output rises steeply with increase of temperature. However, towards the higher temperatures, the results are somewhat irregular, and for this reason the experimental procedure was modified in the hope that a well-defined curve relating temperature and rate of output might be obtained. Accordingly in all the experiments described below the organisms were observed at 15° C., and then at a selected experimental temperature, and finally at 15° C. again.

Decrease of temperature, in experiments carried out as just indicated, was accompanied by reduction in the rate of vacuolar output. Although the maximal vacuolar diameter was greater at low temperatures, and very markedly so in the region of 0° C., this was more than counteracted by the much longer interval between successive systoles (Table 1). In the region of 0° C. the rate of output was reduced, in experiments carried out with the hanging-drop method, to just under 20% of its value at 15° C. However, the contractile vacuole continued to operate even when ice crystals formed in the hanging drop (after the addition of freezing mixture to the water-bath), and was only stopped when the organism was destroyed by the complete freezing of the external medium. Except in cases of complete freezing, restoration of the temperature to 15° C. led to a rapid recovery of the rate of output to a level of the same order as that maintained initially at that temperature.

Increase of temperature (above 15° C.) up to about 34° C. was accompanied by an increase of vacuolar output (Fig. 2). There was always an increase in vacuolar frequency, and near the upper end of the temperature range there was also a considerable increase in ultimate diameter of the contractile vacuole, so that the rate of output was increased by several-fold. In an experiment in which a *Carchesium* was subjected to 34° C. the considerable immediate increase in rate of output was only maintained for a few minutes; then the contractile vacuole slowed down, the body swelled, and death ensued. Except after such extreme treatment the rate of vacuolar output decreased rapidly on restoration of the temperature to 15° C. The final level was usually slightly below that of the first period at 15° C. In all these experiments the change from one temperature to another took several minutes. The changes in rate of vacuolar output appeared to accompany closely the changes in temperature, but it would not have been possible to detect a slight lag.

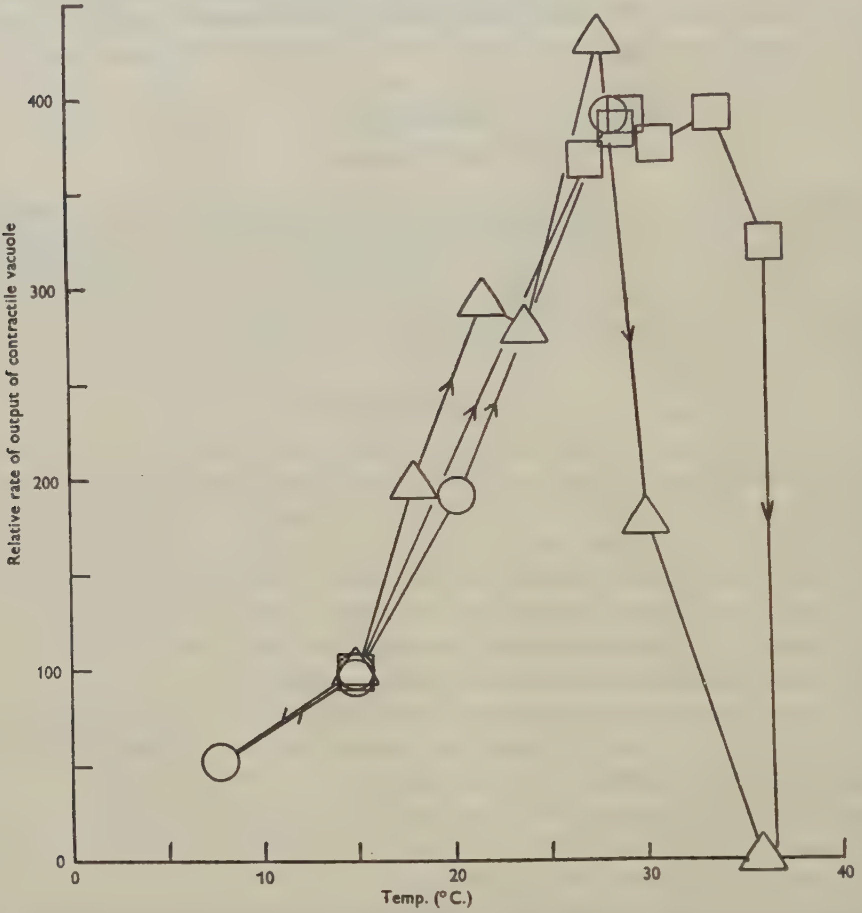

Fig. 1. The effect of temperature on the rate of output of the contractile vacuole of the fresh-water peritrich *Carchesium aselli*. Results of these experiments in which three single organisms (○, □, △) were each exposed to a series of different temperatures.

The relation of mean rate of vacuolar output to temperature, as indicated by these experiments, is shown in Fig. 3. Here each plotted point represents one experiment, the rate of vacuolar output at the test temperature (that is during the

Table 1. *Effects of temperature on activity of contractile vacuole of the fresh-water peritrich* Carchesium aselli *in pond water*

Date	Temp. (° C.)	No. of readings	Mean max. diam. (μ)	Mean duration (sec.)	Rate of vacuolar output (μ^3/sec.)	Rate of output (%) (100 at 15° C.)	Duration of exposure to stated temperatures (min.)
10. v. 46 *b*	15	13	4·5	7·9	6·0	100	2
	0	20	6·3	119·8	1·1	18	56
	15	14	4·5	8·0	6·0	100	2
3. vi. 46 *a*	15	14	3·9	8·2	3·8	100	5
	0	10	5·2	103·2	0·7	18	30
	15	22	3·9	9·2	3·4	89	15
10. v. 46 *a*	15	9	5·1	8·6	8·1	100	2
	1	5	7·6	164·7	1·4	17	14
	15	17	5·3	7·9	9·9	122	3
1. v. 46 *a*	15	26	4·6	7·9	6·4	100	21
	8	12	5·3	27·4	2·8	44	24
13. iv. 46	15	18	5·1	11·9	5·8	100	18
	8·5	11	5·4	43·6	1·9	33	17
	15	10	4·5	11·1	4·3	74	3
14. iv. 46	15	25	4·4	10·4	4·2	100	36
	9	13	4·9	33·8	1·8	43	38
	15	23	4·7	9·9	5·5	131	30
23. iv. 46 *b*	15	28	5·1	7·4	9·4	100	25
	15	32	5·2	7·7	9·6	102	29
	15	32	5·3	7·5	10·4	111	33
	15	34	5·3	7·3	10·7	114	30
4 iv. 46	15	28	4·7	10·0	5·4	100	21
	15	35	4·7	10·0	5·4	100	32
	15	22	4·4	8·8	5·1	94	44*
12. iv. 46	15	34	4·3	7·1	5·9	100	44
	22	35	4·3	4·3	9·7	165	35
	15	21	4·2	7·3	5·3	90	25
6. iv. 46	15	23	5·6	17·3	5·3	100	24
	22	26	5·5	9·5	9·2	174	39
	15	31	5·5	17·3	5·0	94	30
23. iv. 46	15	11	5·8	12·9	7·9	100	16
	23	11	5·6	5·5	16·4	208	8
	15	16	5·8	12·8	8·0	101	16
5. iv. 46 *b*	15	27	5·9	14·7	7·3	100	26
	24	37	5·9	7·0	15·3	210	55
	15	29	5·9	15·8	5·8	79	78
12. iv. 46 *b*	15	29	4·3	7·4	5·6	100	22
	25	24	4·6	4·6	11·1	198	17
	15	22	4·5	11·6	4·1	73	20
	25	22	4·8	4·5	12·9	230 (315)	16
30. iv. 46 *a*	15	29	4·7	9·3	5·8	100	16
	27	18	4·7	4·0	13·6	235	17
	15	31	4·6	12·5	4·1	71	22
30. iv. 46 *b*	15	26	4·9	9·4	6·6	100	20
	31	36	6·1	4·1	29·0	439	20
	15	36	6·0	25·4	4·4	67	38
29. iv. 46	15	22	5·0	13·2	5·0	100	21
	31·5	10	5·8	3·8	26·9	538	9
1. v. 46 *b*	15	10	4·8	10·1	5·7	100	3
	32·5	7	7·0	6·4	28·1	493	4
	15	10	6·5	50·9	2·8	49	8
2. v. 46	15	18	4·3	8·2	5·1	100	4
	34	8	6·3	3·2	40·8	800	1½†

* Temperature fluctuated somewhat during this period.

† After this period the rate of output progressively decreased and the organism finally blistered and died.

second of the three periods) being expressed as a percentage of that at 15° C. (first period). In this way the results of all the experiments have been scaled to the same level (100%) for the initial rate of output at 15° C. This procedure is justified by the fact that the points lie close to a smooth curve and that where points represent independent results for the same temperatures they lie very close together.

The curve plotted in Fig. 3 indicates a Q_{10} for rate of vacuolar output of about 2·5–3·2. If the fit of these data to the form demanded by the Arrhenius equation is tested by a plot of the logarithm of the rate of vacuolar output against the reciprocal of the absolute temperature, the points are found to be scattered not unreasonably about a straight line over the temperature range 0–30° C.; the value for μ is about 17,000.

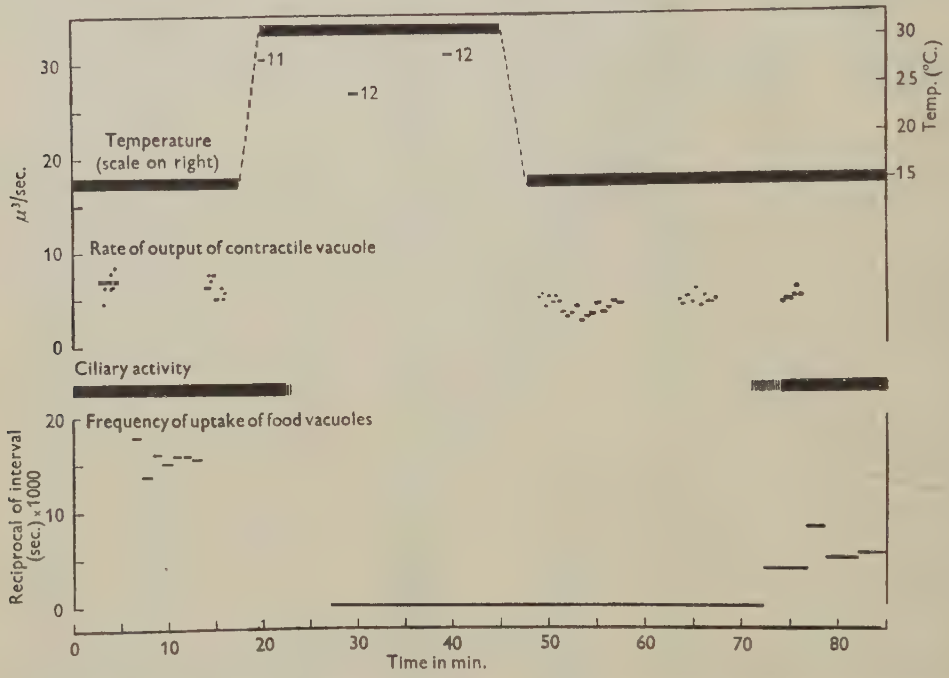

Fig. 2. The effect of exposure to a temperature of 31° C. on the rate of output of the contractile vacuole, the state of activity of the adoral cilia, and the frequency of uptake of food vacuoles, in the fresh-water peritrich ciliate *C. aselli*. At high temperature it was not possible to measure the times of systole and the maximal diameters for the same vacuolar cycles, so that a series of each was measured; the points plotted in this case represent estimates of rates of output averaged from readings of maximal diameter and of vacuolar duration to the number indicated.

Food vacuoles and ciliary activity

Decrease in temperature was accompanied by a decrease in frequency of uptake of food vacuoles (Table 2). At, or slightly below, 0° C. no food vacuoles were taken up, and the cilia stopped. On return to a temperature of 15° C. the frequency of uptake of food vacuoles was restored to a level of the same order as that in the initial period at that temperature, and, after exposure to 0° C., the cilia resumed their activity. In one case, after 0° C., recovery of food-vacuolar and ciliary activity was delayed for some minutes; in the other cases no such delay was observed.

Increase of temperature up to 24° C. was accompanied by an increase in frequency of uptake of food vacuoles. On a return of the organism to a temperature of 15° C. the frequency of uptake of food vacuoles was reduced to a value in most cases somewhat lower than the original for that temperature. No food vacuoles were taken up at temperatures above about 30° C., and the disk cilia stopped beating.

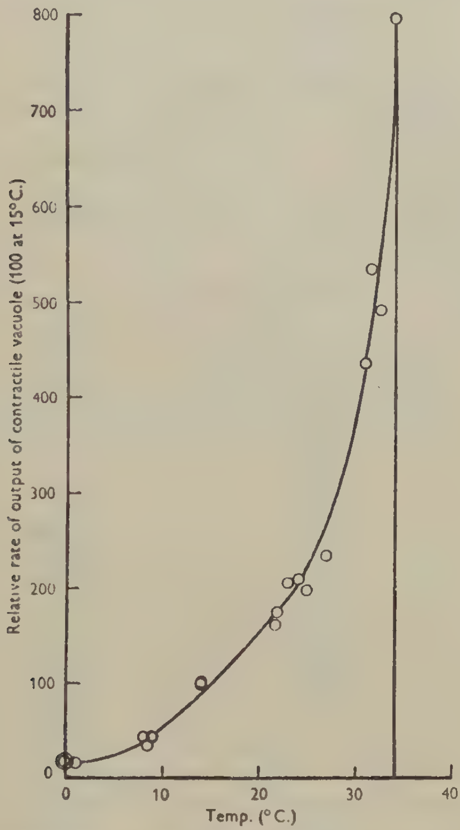

Fig. 3. The relation between temperature and rate of output of the contractile vacuole in the fresh-water peritrich *C. aselli*. This graph is based on the data given in Table 1. For each experiment the rate of vacuolar output at 15° C. has been taken as 100.

In such cases on return of the organism to a temperature of 15° C. recovery of food-vacuolar and ciliary activity was delayed for some time (Fig. 2).

The relation between frequency of uptake of food vacuoles and temperature is shown in Fig. 4. Frequency is expressed as $10^3 \times$ the reciprocal of the mean interval between times of uptake of successive food vacuoles. Since no significant differences could be found in the sizes of food vacuoles taken up at different temperatures, this curve approximately represents the relation of rate of uptake of water by food vacuoles to temperature.

Table 2. *Effect of temperature on frequency of uptake of food vacuole by the fresh-water peritrich* Carchesium aselli *in pond water*

Date	Temp. (° C.)	No. of readings (i.e. intervals)	Mean interval (sec.)	Standard error (applicable to mean interval)	$10^3 \times$ reciprocal of mean interval	Relative frequency (100 at 15° C.)	Condition of cilia on disk
5. v. 46	15	4	141·3	7·7	7·1	100	Active
	0	No food vacuoles	∞	—	0·0	0	Stopped
	15	*	—	—	—	—	Renewed activity after 10 min. frequent intermissions
4. vi. 46	15	5	96·6	6·6	10·4	100	Active
	0	No food vacuoles	∞	—	0·0	0	Stopped
	15	*	—	—	—	—	Active immediately
4. vi. 46 *b*	15	5	84·2	6·4	11·9	100	Active
	0	No food vacuoles	∞	—	0·0	0	Stopped except for occasional flicker
	15	5†	140·6	6·2	7·1	60	Active immediately
1. v. 46 *a*	15	7	75·0	2·7	13·3	100	Active
	8	3	164·3	3·5	6·1	46	Active
16. iv. 46	15	10	82·1	3·4	12·2	100	Active
	8	3	206·0	16·7	4·9	40	Active
	15	5	96·0	6·1	10·4	85	Active
14. iv. 46	15	9	129·8	8·1	7·7	100	Active
	9	4	297·5	9·9	3·4	44	Active
	15	7	108·7	4·6	9·2	119	Active
23. iv. 46 *b*	15	18	57·1	2·0	17·5	100	Active
	15	19	52·6	1·3	19·1	109	Active
	15	22	61·1	1·3	16·4	94	Active
	15	8	60·8	1·2	16·4	94	Active
6. iv. 46	15	3	194·0	8·3	5·2	100	Active
	22	6	133·5	26·5	7·5	144	Active
	15	4	228·0	44·3	4·4	100	Active
12. iv. 46	15	11	71·3	1·5	14·0	100	Active
	22	16	62·5	1·3	16·0	114	Active
	15	6	102·5	5·9	9·8	70	Active, but brief intermissions
23. iv. 46	15	8	88·2	3·7	11·3	100	Active
	23	5	64·6	2·2	15·5	137	Active
	15	7	93·0	5·4	10·8	96	Active
26. iv. 46	15	12	62·4	1·9	16·0	100	Active
	24	11	42·7	2·4	23·4	146	Active
12. iv. 46 *b*	15	14	50·6	1·2	19·8	100	Active
	25	7	53·0	2·8	18·9	95	Active
	15	9	81·6	3·6	12·3	62	Active
30. iv. 46 *a*	15	7	79·7	2·0	12·5	100	Active
	27	8	70·0	2·7	14·3	114	Active
	15	6	100·8	6·0	9·9	79	Active
30. iv. 46 *b*	15	7	63·9	1·6	15·6	100	Active
	31	No food vacuoles	∞	—	0·0	0	Stopped
	15	4	—	—	Some recovery after 25 min., see Fig. 2		
29. iv. 46	15	6	76·7	3·4	13·0	100	Active
	31·5	No food vacuoles	∞	—	0·0	0	Stopped

* Some food vacuoles taken up after 20 min.

† Preceded by several vacuoles at longer intervals.

Measurements were made of the volume of the oesophageal sac, which periodically becomes cut off as a food vacuole, in the case of four individuals at 15° C. From these values and from the frequency of uptake in each of these cases it was calculated that the rate of uptake of fluid in food vacuoles was 10–14% of the rate of output by the contractile vacuole.

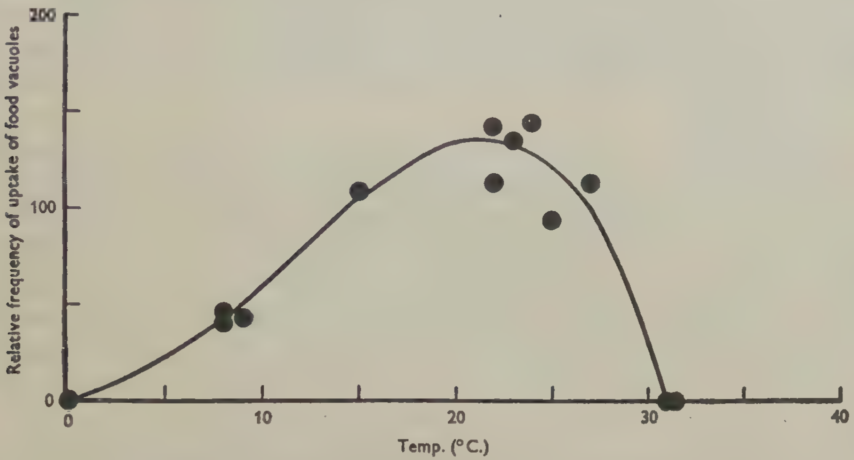

Fig. 4. The relation between temperature and the frequency of uptake of food vacuoles in the fresh-water peritrich ciliate *C. aselli*. This graph is based on the data given in Table 2. For each temperature the frequency, calculated as the reciprocal of the mean interval between successive uptakes of food vacuoles, has been taken as 100 at 15° C.

Body volume

No significant changes were observed in the length or breadth of the body during any of the experiments described above (except for the swelling noted at excessively high temperature). A few additional experiments of longer duration were carried out, in which the temperature control was slightly less rigid. Results are summarized in Table 3. In no case was there any suggestion of a change in body volume, although sometimes there was a slight tendency towards shortening and widening of the body at higher temperatures.

Table 3. *The effect of temperature on the activity of the contractile vacuole of the fresh-water peritrich* Carchesium aselli

Summary of longer experiments. The osmotic pressure of the pond water, obtained by measurement of the freezing-point, was 0·17 atmospheres at 15° C.

Date of experiment	Experimental temperature (° C.)	Duration for which organism was at experimental temperature (min.)	Relative rate of output at experimental temperature (original rate at 15° C. as 100)
27. v. 47	10	133	Average 29 (no detectable drift)
2. x. 47	29	120	About 390 falling to about 260
12. v. 47	28	120	About 530 falling to about 420
28. x. 47	28	287	About 475 falling to about 300
4. xi. 47*	28	151	About 300 falling to about 210

* The medium for this experiment was 10% pond water in glass-distilled water; for all the others it was pond water.

DISCUSSION

Temperature and the exchange of water

The chief components of the water exchange of a peritrich ciliate are osmotic uptake and the output of the contractile vacuole (p. 406). The part played by food vacuoles, which is in any case of minor importance, becomes reduced to zero at unnaturally high temperatures (30° C. and over; see Fig. 4). Accordingly, the effects of temperature will be considered in relation to the balance between osmotic uptake and vacuolar output.

No changes could be detected in the body volume of *Carchesium aselli* exposed to various temperatures near the limits of the biological range for periods of 2 hr. (and in one case $4\frac{1}{2}$ hr.) even though the rate of output of the contractile vacuole was greatly altered. Clearly the maintenance of a constant body volume would require a simultaneous compensatory change in the rate of uptake of water from the external medium. The rate of osmotic entry of water into the body is presumably determined by the difference of osmotic pressure between the inside and the outside and by the permeability of the body surface to water. An appropriate alteration in one or both of these would produce the necessary change in rate of uptake of water.

However, before we can conclude that such an alteration has taken place, it is necessary to consider both the rate and the magnitude of the change in body volume which would otherwise have been expected; it must be shown that this change in body volume could have been detected during the experiments. Various factors might tend to retard and to reduce such a change—for instance, the presence of osmotically inactive non-aqueous material within the body of the organism or of non-penetrating solutes in the external medium.

The steady state of water balance may be expressed as

$$\text{Rate of output} = \text{Rate of uptake} = (\text{approx.})\ kA\,(P_0 - p),$$

where P_0 = original osmotic pressure of organism, p = osmotic pressure of external medium, A = surface area of water-permeable membrane, and k is a constant representing permeability to water and theoretically characteristic of the membrane.

Let us suppose that the rate of output is changed, but that the content of solute in the body and the permeability of the water-permeable membrane remain unaltered. The steady state is now upset. The body volume changes, and with it the internal osmotic pressure of the organism, until a new steady state is attained at a level determined by the new rate of output. During this readjustment the momentary rate of change of body volume will be equal to the rate of uptake of fluid minus the rate of output of the contractile vacuole (swelling being regarded as a positive change):

$$dV/dt = kA\,(P-p) - xkA\,(P_0-p) = kA\,[P-(p+xP_0-xp)],$$

where V = volume of organism, P = osmotic pressure of organism at time t, and x = new rate of output divided by original rate of output. This relation is similar in form to that deduced by Northrop for the swelling of marine eggs in hypotonic solutions. Northrop's equation, discussed in the review by Lucké & McCutcheon (1932), is given there as $dV/dt = C\,(P - P_{ex})$, in which P = osmotic pressure of egg at time t, and C is a constant depending upon the surface area and the permeability to water of the membrane. The term P_{ex} in Northrop's equation represents the osmotic pressure of the external solution with which the egg finally comes into equilibrium: it corresponds to the term $(p+xP_0-xp)$ for the peritrich, this being the ultimate internal osmotic pressure at which the body volume would become stabilized under the new rate of vacuolar output. Thus in both cases the rate of change of volume is proportional to the difference between the momentary and the ultimate internal osmotic pressure, and in both cases the total content of solute is assumed to remain unaltered.

For the integration of Northrop's equation a relation is necessary between internal osmotic pressure and cell volume. The simplest integrated form, derived on the assumption that the product of internal osmotic pressure and cell volume is constant (Lucké & McCutcheon, 1932, p. 103, equation 3*a*), has been adjusted for the present purpose (see under Fig. 5) to allow for non-aqueous materials within the body; other more complicated corrections are inappropriate to the present circumstances. The change in the osmotic pressure of the dilute internal solution with temperature will be small over the range under consideration, and has therefore been neglected.

Sample theoretical shrinkage curves, constructed on the assumption of a change in rate of vacuolar output without change in the permeability of the body surface or in the solute content of the body, are given in Fig. 5. They apply to a *C. aselli* of average size. Since it is not at present possible to say how much non-aqueous material there is within the body, curves have been calculated for a range of possible quantities. However, it should be pointed out that, when *C. aselli* is transferred to a relatively strong (e.g. molar) solution of sucrose, the body shrinks greatly and becomes strongly wrinkled. The amount of such material must therefore be relatively small, and in any case is not so great as to mask osmotic shrinkage when the difference of concentration is substantial. In other respects the values used for substitution in the equation, and quoted below Fig. 5, are extremes such as would give the slowest changes of body volume.

From the curves given in Fig. 5 it is clear that, on the assumptions on which the treatment described above is based, an increase in rate of vacuolar output to 300–400% would produce a very marked shrinkage well within the durations of the experiments. For instance, with the vacuolar output at 400%, and even with a non-aqueous volume of 25%, *C. aselli* would be expected to shrink by 50% of its total volume within about 15 min. Even with a greater quantity of non-aqueous materials equilibrium would be approached relatively quickly. Since no change in the body volume was observed it must be concluded that some condition, assumed to be constant in the foregoing treatment, must in fact change with temperature.

The increase in the rate of osmotic uptake, which accompanies an increase of temperature, might be brought about (without change of body volume) either by an increase in the number of solute molecules within the body or by an increase in the 'permeability' to water of the membrane through which uptake occurs. The former effect might result from an increased accumulation of metabolites or from an increased ionization of cell proteins. On the other hand, in favour of an increased permeability, it is known (Lucké & McCutcheon, 1932) that the osmotic swelling of *Arbacia* eggs proceeds more rapidly at higher temperatures, the calculated 'permeability' to water having a Q_{10} of from 2·5 to 3·1; this is of the same order as that found for the vacuolar output of *Carchesium aselli*. Evidence will be presented in the next paper of this series that the effect of temperature is on the permeability of the membrane, and that the resulting change in osmotic stress in turn provokes a change in the rate of vacuolar output.

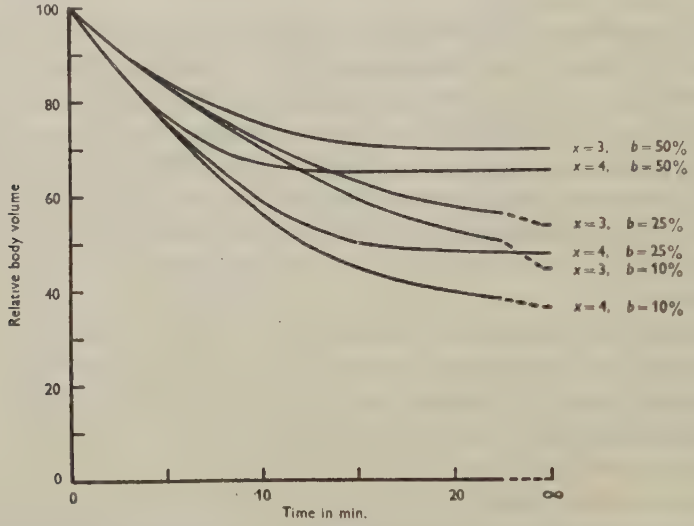

Fig. 5. Theoretical shrinkage curves for the hypothetical case of a ciliate which undergoes an increase in the rate of vacuolar output without change of any other conditions influencing the water balance. These curves have been calculated from the relation

$$C\,.\,P_0\,(V_0-b)\,t=(V_e-b)^2\,l_n\,\frac{V_0-V_e}{V-V_e}+(V_e-b)\,(V_0-V),$$

when C = permeability constant × surface area (taken as 0·1 μ^3/μ^2/atm./min. × 3600 μ^2), P_0 = original internal osmotic pressure (taken as 0·04 × 25 atm.), V_0 = original volume (taken as 17,000 μ^3), b = volume of osmotically inactive non-aqueous material within the organism (values for which are specified above), V_e = volume after attainment of new steady state, calculated from the relation

$$P_0\,(V_0-b)=(xP_0+p-xp)\,(V_e-b),$$

where x is the new rate of vacuolar output divided by original rate of vacuolar output, p is the osmotic pressure of external medium (= 0·17 atm.) and V is the volume at time t.

General effects of temperature

The increase in rate of output of the contractile vacuole with increase of temperature may be regarded, by those who hold that contractile vacuoles eliminate nitrogenous waste, as a necessary physiological response to an increase of metabolic rate. There is little evidence by which this view may be tested. Attempts by Weatherby (1929) to determine the approximate concentration of urea in the vacuolar fluid of *Spirostomum* appear to indicate that most of the urea formed in the organism must be eliminated in another way. On the other hand, the body surface of marine 'limax' amoebae (Pantin, 1931) and of marine peritrich ciliates (Kitching, 1936) is relatively impermeable to urea in the external medium. Much more information is needed before any conclusions can be reached. Nor must it be assumed that 'the contractile vacuole' performs the same functions in the same degrees throughout the sub-kingdom of Protozoa. It may be added, in view of the great turnover in water brought about by vacuolar activity, that it might be expected that the contractile vacuole would exercise some power of retention or resorption of solutes; and, as a speculation, that this active process is likely to be selective.

The effects of temperature on the ciliary activity and on the uptake of food vacuoles of *Carchesium aselli* are strikingly similar. Both are inhibited at temperatures above 30° C., and on return to a more normal temperature there is a simultaneous delayed recovery. Both also are reversibly inhibited at or about 0° C. On the other hand, the contractile vacuole operates, for a limited time at any rate, at temperatures slightly above 30° C. and at 0° C. All this suggests that there is either some fundamental similarity in mechanism or some co-ordination between the post-oesophageal fibrils, which are believed to be responsible for the uptake of food vacuoles (Bozler, 1924; Mast & Bowen, 1944), and the cilia of the disk. It has been shown (Kitching, 1938*b*) that the initial movements of the food vacuoles of peritrich ciliates can only be accounted for by the action of a contractile process taking place in the surrounding protoplasm—a process which is quite probably the function of the oesophageal fibrils. Their activity is therefore perhaps closely akin to that of cilia, so that they might in any case respond in the same way as the cilia to extremes of temperature. Apart from this, the response of the contractile vacuole to high temperatures may differ from that of other organelles because of the special effect of increased osmotic stress, which is likely to elicit the greatest possible output until the mechanism breaks down.

SUMMARY

1. The rate of output of the contractile vacuole in a fresh-water peritrich ciliate (*Carchesium aselli*) varies with temperature with a Q_{10} of about 2·5–3·2, or a μ of about 17,000, over the range 0–30° C.

2. There is a slow decline in output during exposure for several hours to high temperatures (25–30° C.). At still higher temperatures (34° C.) a high rate of output is maintained for a few minutes, but swelling and death rapidly ensue.

3. The frequency of uptake of food vacuoles also varies with temperature, increasing from 0 to about 24° C., but decreasing at higher temperatures. At about 0° C. and at temperatures above about 30° C. no food vacuoles are taken up and the adoral cilia remain extended and motionless.

4. No change in body volume could be detected during exposure to high temperatures (25–30° C.) for two or more hours, even though the rate of vacuolar output was increased to three or four times its normal level at 15° C. It is concluded that the rate of uptake of water from the outside medium must have been increased correspondingly.

5. It is suggested that temperature affects the permeability of the organism to water, and that the rate of vacuolar output is adjusted accordingly, although on the evidence so far presented other explanations are possible.

It is a pleasure to thank Prof. J. E. Harris for much valuable advice given during the course of this work. Specimens of the peritrich ciliate *Carchesium aselli* were kindly examined and determined for me by Mr A. G. Willis.

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THE PHYSIOLOGY OF CONTRACTILE VACUOLES

VI. TEMPERATURE AND OSMOTIC STRESS

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(With Six Text-figures)

INTRODUCTION

Increase of temperature, within reasonable biological limits, causes an increase in the rate of output of the contractile vacuole of certain peritrich ciliates without, over a limited period, detectably altering their body volume (Kitching, 1948). It must therefore be concluded that it also produces some corresponding change by which the rate of uptake of water by the animal is immediately and equally increased. Two possibilities have been suggested: the permeability of the body surface, or part of it, to water might be affected by temperature, and so might the concentration of dissolved substances within the organism.

The analysis of the kinetics of osmotic swelling in sea-urchin eggs (reviewed by Lucké & McCutcheon, 1932) has provided a basis for the estimation of the permeability of their surface to water. For the purpose of comparable experiments on peritrich ciliates it seemed better to use a marine than a fresh-water form. Shrinkage cannot be followed experimentally in peritrichs owing to the wrinkling of the body surface; and fresh-water forms are already adapted for existence in a medium of very low osmotic concentration. Moreover, the course of swelling of a marine peritrich in dilute sea water is unlikely, in its initial stages, to be modified by the contractile vacuole; this operates at a low rate except at considerable dilutions (Kitching, 1934). Owing to the large size of sea-urchin eggs, the process of osmotic swelling in their case is not too rapid to be followed conveniently; most peritrich ciliates are much smaller, so that swelling would be likely to take place too quickly to be easily recorded. In view of these considerations the relatively large marine peritrich *Vorticella marina* was chosen for this part of the present work. The results obtained in this way may be applied with reasonable confidence to the fresh-water form, *Carchesium aselli*, discussed in the previous paper.

In previous measurements of the internal osmotic pressure of *C. aselli* (Kitching, 1938) the activity of the contractile vacuole, which might interfere with the results, was suppressed by means of cyanide, and the concentration of sucrose in the external medium which would then maintain the body at constant volume was determined. This procedure is open to the possible objection that cyanide might itself affect the concentration of metabolites such as lactic acid within the organism, and so might change the internal osmotic pressure, although there is no evidence to suggest that this actually occurred. Accordingly, the internal osmotic pressure has now been re-investigated by means of a study of the relations of body volume and rate of vacuolar output to the concentration of sucrose in the medium (without cyanide), and the method has also been applied at high temperature (30° C.). The results of these experiments, together with those on the permeability to water of *Vorticella marina*, provide the evidence necessary for an explanation of the effect of temperature on the osmotic uptake of water.

Consideration of the problem outlined above immediately suggested another, for the discussion of which some of the same evidence is also relevant. Does temperature influence the rate of vacuole output and of osmotic uptake equally, simultaneously, and independently? Or does temperature first influence the rate of uptake, thereby disturbing the water balance in such a way as to produce a very small change in body volume, to which the contractile vacuole is sensitive and responds appropriately? If the former alternative were true, its investigation might offer an interesting approach to the further study of the mechanism of vacuolar activity. However, the latter explanation would conform more closely to the usual pattern of physiological co-ordination in animals.

Although the unequivocal testing of these two alternative hypotheses is difficult, useful evidence can be obtained indirectly. If the organism is sensitive to very small changes in internal hydration (or some other closely related condition), then it should respond equally well to these internal changes whether they are due to changed permeability, to changed internal osmotic pressure, or to changed external osmotic pressure. The capacity of the organism to regulate vacuolar activity (and so body volume) in the face of variable osmotic stress imposed by changes of external solution at constant temperature might be expected approximately to equal its capacity to do so in the face of variable osmotic stress resulting from change of temperature. If this were found to be true there would at least be no reason to postulate an independent and controlling effect of temperature on vacuolar activity.

This question has been tested by experiments in which *Carchesium aselli* has been subjected at constant temperature to conditions calling for reduced vacuolar output and also for greatly increased vacuolar output. For the former the experiments with solutions of sucrose, outlined above, are appropriate. For the latter, the organism was immersed in solutions of ethylene glycol, which penetrates, or escapes from, animal cells at a moderate rate (Jacobs & Stewart, 1932). In this way the internal osmotic pressure of the organism could be built up to such a level that on return to water not containing this substance a considerable osmotic gradient was temporarily imposed. The remarkable capacity of the organism to regulate its body volume in the face of these osmotic gradients is described in the latter part of this paper.

METHODS

The marine peritrich, *Vorticella marina*, growing on the green filamentous alga *Cladophora*, was received by post from Plymouth, and kept in large jars of sea water in a room at a temperature not exceeding 15° C. It was used for experiments on the effect of temperature on the rate of osmotic swelling. The fresh-water peritrich *Carchesium aselli* was found in a pond in the University grounds on the legs of the isopod *Asellus aquaticus*. It was used for the experiments on osmoregulatory capacity.

For these various experiments it was necessary to transfer the organisms rapidly from one solution to another at any desired temperature while under continuous observation. The apparatus for irrigation of sessile organisms at controlled temperatures (Kitching, 1934) was modified in certain particulars. This apparatus, as previously described, consists of a glass tube (the 'supply tube') passing through a water-jacket at constant temperature. The supply tube opens by a capillary aperture at the edge of the cover-glass under which the organism is mounted, and delivers a continuous supply of the appropriate medium. The liquid is drained away at the other side of the cover-glass into a funnel by means of filter-paper. For the purpose of the present investigation a loop of copper tubing, for the circulation of carbon dioxide snow, was inserted into the water-jacket in order that low temperatures might be attained more quickly. For the lowest temperatures (around 0° C. with sea water) alcohol was added to the water in the water-jacket to depress the freezing-point. The liquid was then chilled until sufficient had frozen around the copper tubing to give a good reserve of cooling power. With air bubbled through the liquid in the water-jacket to stir it, and with water flowing down the central supply tube at a constant rate, a steady low temperature could be maintained on the microscope slide with very little trouble. The temperature under the cover-glass was as usual measured with a thermocouple.

Quick change of solutions under the cover-glass was effected by the addition of a three-way stopcock to the supply tube before its entry into the water-jacket. The two different solutions to be used (e.g. sea water and dilute sea water) were led by rubber tubing to the stopcock. For the experiments on osmotic swelling a bubble of air was introduced into the limb of the stopcock through which the second solution was supplied, so that when the cock was turned this solution travelled down the supply tube separated by a column of air from the first solution. In this way it was possible to observe the instant at which the second solution reached the cover-glass. In the case of the experiments on osmotic swelling a few drops of a thin suspension of the red powder Scarlet Ancolor Y were injected through the rubber tubing into the second solution at the stopcock and immediately behind the air bubble. Instantaneously on arrival of the second solution at the edge of the cover-glass the red particles were distributed all over the field of vision of the microscope and streamed by rapidly. Thus immediate and thorough replacement of the first solution by the second was always established. The red particles were not used in the other experiments (with sucrose or ethylene glycol), but the flow was brisk enough to assure a quick change of solution under the cover-glass.

Estimation of the body volume of *Vorticella marina*, for the experiments on osmotic swelling, was carried out according to the general principles already described (Kitching, 1934), but with certain modifications. It is necessary that the organisms be symmetrical about an axis of rotation in order that a side view in the plane of the central axis may provide the information necessary. A normal expanded *V. marina* departs rather widely from this condition, nor can any allowance be made for the oral depression, but when contracted it is compact and symmetrical. Accordingly, the organism was caused to contract by a sharp knock on the bench, and it was then quickly photographed with a Leica microscope camera. This procedure was necessary during the first part of an experiment when the organism was in 100% sea water. After it had been transferred to dilute sea water, during the early stages of swelling no external stimulation was necessary to obtain contraction; later the heavily swollen organism was symmetrical, and could contract only very slightly. Since the stalk of *V. marina* is long and very flexible, the continuous stream of liquid was sufficient to hold the organism accurately in side view. After the photographic film had been developed each negative was projected on to white paper and the outline of the organism was traced in pencil. The central axis was established by a straight line drawn from the centre of the base through the mid-point of the widest diameter. The outline of each half of the figure was then traversed at least twice with a moment integrator, and the readings of first moment were averaged to give an estimate of the body volume. A similar procedure has been used by Adcock (1940) for estimation of the volume of gregarines.

Observations of contractile vacuoles and food vacuoles was carried out (in the experiments with sucrose and ethylene glycol) as in previous papers of this series (Kitching, 1934).

RESULTS

Effect of temperature on rate of osmotic swelling

In ten experiments photographic records were successfully obtained of the body volume of *V. marina* before and during osmotic swelling. Four of these experiments were carried out at high temperature (24–29° C.), two at intermediate temperature (14–17° C.), and four at low temperature (1–3° C.). The swelling was induced by a rapid change from 100 to 25% sea water. The results are given in Fig. 1.

The initial rates of swelling are shown in Fig. 1 by lines drawn tangentially to the swelling curves. These may be used as a measure of the permeability of the surface, on the assumption of a constant surface area of permeable membrane and of a constant solute content of the body. The figures on the key to permeability in the bottom right quarter of the figure are given in cubic microns per square micron of body surface per atmosphere per minute, on the assumption that the whole body surface is equally permeable. (They may be suitably corrected if this assumption should be proved wrong.) In view of the fact that for a given permeability the slope will vary with the size of the organism, the key provides for two sizes of *Vorticella*.

In some of the experiments after the organism had undergone some swelling the pellicle became lifted up in blisters. This happened in all cases at the higher temperatures. For each occasion when the organism was blistered the circle on the graph is marked with a short vertical line above it. Such readings do not include the volumes of blisters, which could not be ascertained; both for this reason and because salts are likely to have escaped, these readings are to be regarded as too low from the point of view of the kinetics of osmosis.

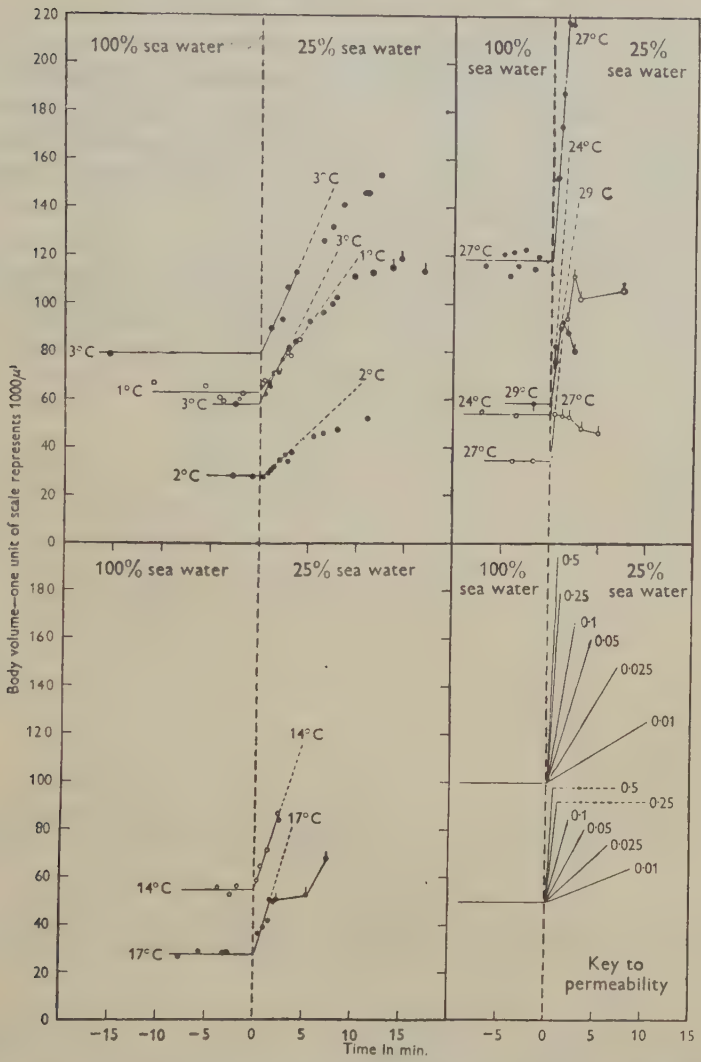

Fig. 1. Rate of swelling of *Vorticella marina* on transfer from 100 to 25 % sea water. Results are given for low temperatures (top left), intermediate temperatures (bottom left), and high temperatures (top right). Lines drawn at a tangent to the swelling curves indicate the initial rates of swelling. Where the point is marked with a short vertical line the organism was blistered. The key (bottom right) shows the theoretical initial rate of swelling for various permeabilities of the body surface, in cubic microns of water passing per square micron of body surface per atmosphere per minute, on the assumption that the body surface is uniformly permeable.

The initial slopes of the swelling curves are greatest at the high temperatures, corresponding to permeabilities of 0·25–0·5 μ^3/μ^2 atm./min. on the key; they are intermediate at intermediate temperatures, at about 0·1 on the key; and they are lowest at the low temperatures, with slopes of around 0·025. These values suggest a Q_{10} of around 2·5–3·2, but the method used is somewhat crude and too much reliance must not be placed on the figures.

Effect of sucrose solutions on the water balance

Specimens of the fresh-water peritrich *Carchesium aselli* were irrigated at 15 ± 0·5° C. with pond water (pH 7·7–8·0), with one or more solutions of sucrose in pond water, and again with pond water. Observations were made of the dimensions of the body, of the maximal diameters and times of systole of the contractile vacuole, and of the times of uptake of food vacuoles.

Transference of the organism to a dilute solution of sucrose (e.g. 0·01–0·03 M) was rapidly followed by a reduction in rate of output of the contractile vacuole to a new steady value, as illustrated in Fig. 2. The rate of output was reduced to a very low value in 0·05 M-sucrose. The frequency of uptake of food vacuoles was at first little affected by dilute solutions of sucrose, but later became lower; and frequently there were intermissions both of ciliary activity and of uptake of food vacuoles. Food vacuoles formed in solutions of sucrose were sometimes very small. It was noticeable that food vacuoles containing sucrose persisted much longer than those formed in plain pond water, so that the number of food vacuoles visible inside the *Carchesium* increased, until it sometimes became very difficult or even impossible to follow either the food vacuoles or the contractile vacuole. It was at this stage that the intermissions occurred. No significant change could be detected in the dimensions of the body in concentrations of sucrose of up to 0·03 M. In one experiment (Fig. 3) very slight shrinkage may have occurred in 0·04 M-sucrose, and shrinkage was definite in 0·05 M-sucrose. The results of two experiments in which it was possible to observe the effects of a series of sucrose solutions are illustrated in Fig. 3. The results of other experiments are summarized in Table 1.

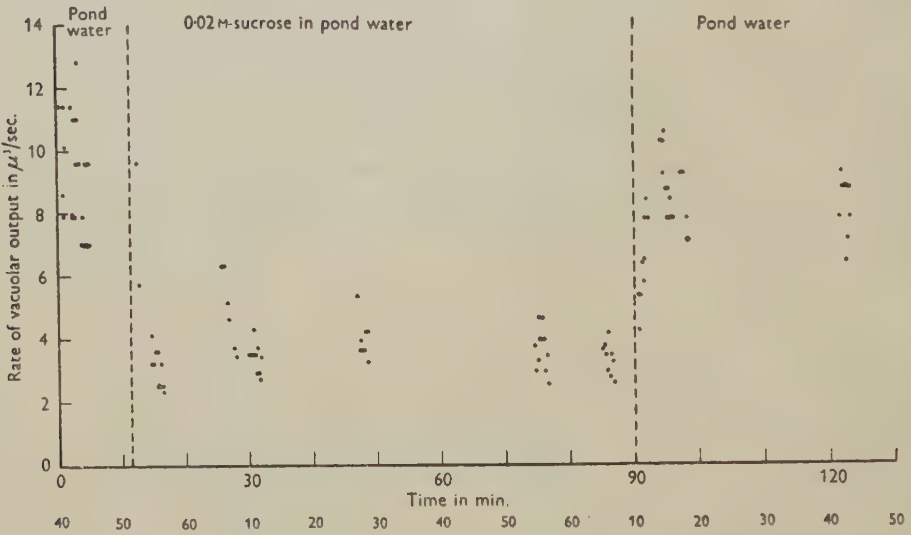

Fig. 2. The effect of a dilute solution of sucrose (0·02 M) in pond water on the rate of output of the contractile vacuole of the fresh-water peritrich *Carchesium aselli*.

In certain experiments there were periods during which no food vacuoles were taken up. Intermission in uptake of food vacuoles and in ciliary activity during the treatment of *C. aselli* with solutions of sucrose have already been mentioned. Sometimes this suppression of ciliary and food-vacuolar activity continued for some time after return of the organism to pond water, as in Exp. 240547 (Table 1). It is noticeable that the contractile vacuole continued to be active in spite of intermission in the uptake of food vacuoles. In Exp. 280547 the organism was not taking up food vacuoles while in pond water at the beginning of the experiment, and did not begin

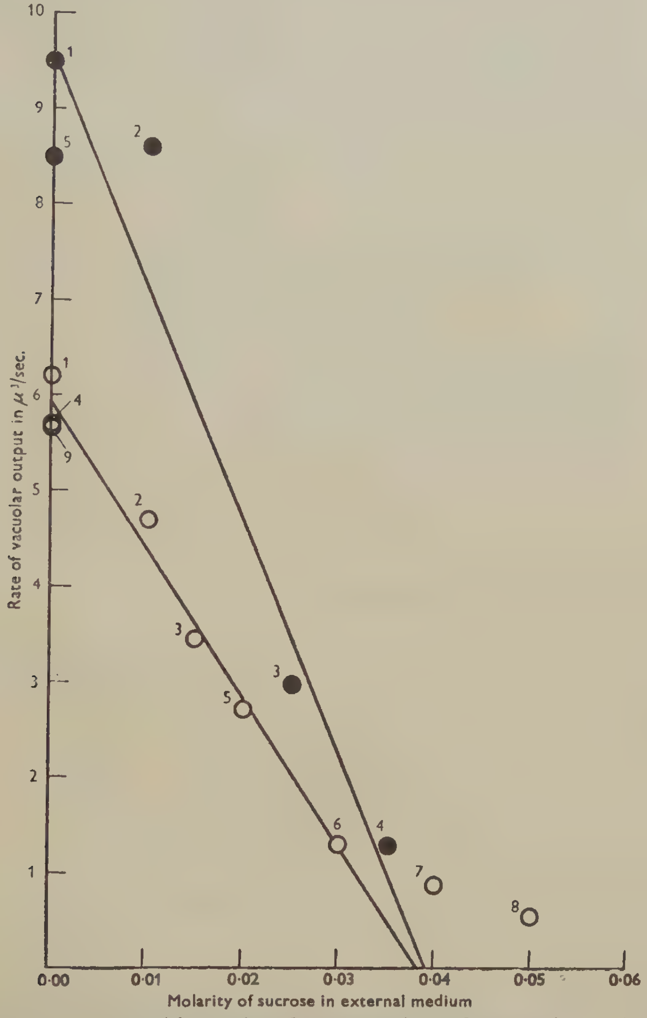

Fig. 3. The effect of treatment with a series of concentrations of sucrose in pond water on the rate of output of the contractile vacuole of the fresh-water peritrich *C. aselli*. The results of two separate experiments are given by clear and black circles respectively. The numbers show the order of the treatment to which the organisms were subjected. The straight lines are drawn through points for concentrations of sucrose at which no shrinkage of the body was detected (i.e not 7 and 8), and are discussed on p. 432.

Table 1. *Effect of sucrose in external medium on rate of output of the contractile vacuole of the fresh-water peritrich ciliate* Carchesium aselli

Reference number of experiment	Concentration of sucrose in medium (molarity)	Duration of treatment (min.)	Number of vacuolar cycles measured	Average rate of output of contractile vacuoles (μ^3/sec.)	Relative rate of output (rate in pond water = 100 %)
130547*a*	—	25	27	10·1	100
	0·01	60	27	5·7	57
	—	14	22	7·7	76
240547	—	19	22	8·5	100
	0·02	78	46	3·6	42
	—	38	26	8·2	96
280547	—	9	12	8·8	100
	0·02	27	22	2·9	33
	—	21	20	6·0	68
060547	—	24	16	7·9	100
	0·03	71	29	3·6	46
	—	19	18	8·5	108
230547*b*	—	16	21	6·6	100
	0·03	52	17	1·2	18
	—	35	20	5·7	86
130547*b*	—	40	32	7·9	100
	0·04	75	17	0·8	10
	—	8	10	7·8	99

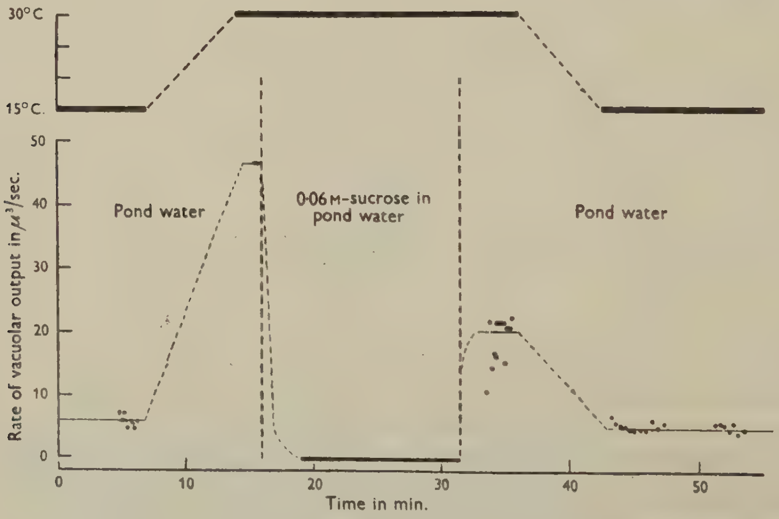

Fig. 4. The combined effects of a solution of sucrose (0·06 M in pond water) and high temperature (30° C.) on the rate of output of the contractile vacuole of the fresh-water peritrich *C. aselli*.

to take them up regularly until it had been for about 15 min. in the sucrose solution. Here again the action of the contractile vacuole does not appear to have been affected.

In three experiments *C. aselli* was irrigated with sucrose solutions while at high temperature (30° C.). The general procedure is illustrated by the records for one of these experiments (Fig. 4), which is typical. *Carchesium* was in this case subjected successively to the following treatments: (1) pond water at 15° C., (2) pond water at 30° C., (3) 0·06 M-sucrose in pond water at 30° C., (4) pond water at 30° C., (5) pond water at 15° C. For one of the other two experiments 0·075 M-sucrose was used, and in the other experiment two strengths of sucrose were given, namely 0·025 and 0·05 M, both at 30° C. In all these experiments elevation of the temperature to 30° C. was accompanied by the usual great increase in the rate of vacuolar output. Treatment at 30° C. with 0·05–0·075 M-sucrose led to a complete stoppage of vacuolar output, accompanied by a marked shrinkage of the body. Treatment at 30° C. with 0·025 M-sucrose led to a reduction in rate of vacuolar output to 24% of that found with pond water at 30° C., or 88% of that for pond water at 15° C. In these three experiments on return of the organisms to pond water there was a recovery of the rate of vacuolar output, at 30° C. and at 15° C., to levels somewhat below those at which it had originally stood at these temperatures. The results of these experiments are summarized in Table 2.

Table 2. *The combined effects of high temperature and of sucrose in the external medium on the rate of output of the contractile vacuole and on the body volume of the fresh-water peritrich ciliate* Carchesium aselli

Reference number of experiment	Temp. (° C.)	Concentration of sucrose in external medium (molarity)	Duration of treatment (min.)	Number of vacuolar cycles measured	Average rate of output of contractile vacuoles (μ^3/sec.)	Note on body volume
130348	15	—	5	14	9·0	—
	30	—	4	10	32·3	No detectable change
	30	0·025	12	24	7·9	No detectable change
	30	—	20	22	30·0	No detectable change
	30	0·05	14	—	0·0	Considerable shrinkage
	30	—	9	23	24·2	Restored approximately to normal
	15	—	4	13	4·3	No detectable change
120348	15	—	4	10	5·6	—
	30	—	5	9	46·6	No detectable change
	30	0·06	16	—	0·0	Probably a slight shrinkage
	30	—	5	9	19·7	Approximately normal
	15	—	16	18	5·0	Approximately normal
110348	15	—	2	10	15·5	—
	30	—	10	12	43·2	—
	30	0·075	10	—	0·0	Considerable shrinkage
	30	—	18	21	32·6	Restored to normal
	15	—	12	16	4·8	No detectable change

Effect of ethylene glycol on the water balance

In ten experiments the fresh-water peritrich *C. aselli* was irrigated with: (1) pond water, (2) a solution of ethylene glycol in pond water, (3) pond water. In all cases the medium was at 15 ± 0·5° C. and pH 7·6–8·0. Observations were made of the dimensions of the body and of the ultimate diameter and times of systole of the contractile vacuole. Typical results are illustrated in Fig. 5.

During irrigation with solutions of ethylene glycol ranging from 0·025 to 0·5 M there was first a depression of vacuolar output, most marked at the highest concentration. Subsequently there was a partial recovery, which was, however, incomplete; at the highest concentrations used (0·5 M) the rate of vacuolar output levelled off at only one-third of its original value, even though equilibration continued for 75 min. At concentrations of ethylene glycol above 0·05 M there was first a shrinkage of the body, but along with the recovery in vacuolar output the animal regained its normal volume.

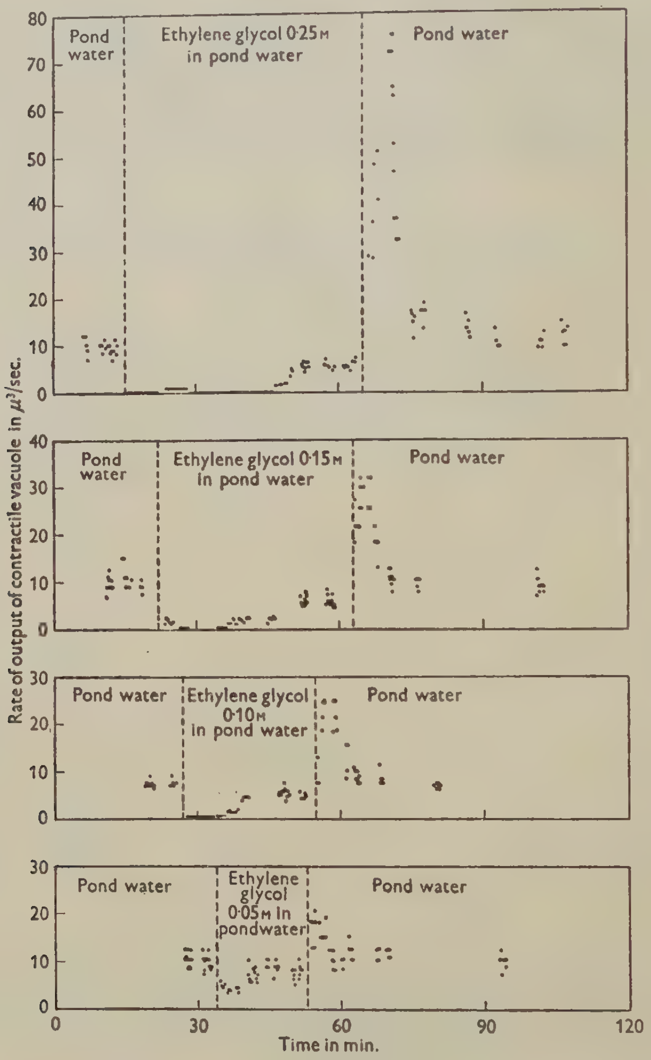

Fig. 5. The effects of treatment with solutions of ethylene glycol in pond water, and of return to pond water after equilibration with a solution of ethylene glycol, on the rate of output of the contractile vacuole of the fresh-water peritrich *C. aselli*.

When the organism was irrigated once again with plain pond water, there was an immediate and great increase in rate of vacuolar output, which was followed by a decline to about the level at which it had originally stood at the beginning of the experiment. In Fig. 6 is shown the relation between the peak rate of vacuolar output, attained immediately after return to pond water, and the concentration of ethylene glycol with which the organism had been equilibrated. There was a considerable swelling of the body on return of the organism to pond water in two cases, namely after treatment with 0·5 and with 0·25 M-ethylene glycol. In all other cases the body volume remained constant as far as could be detected.

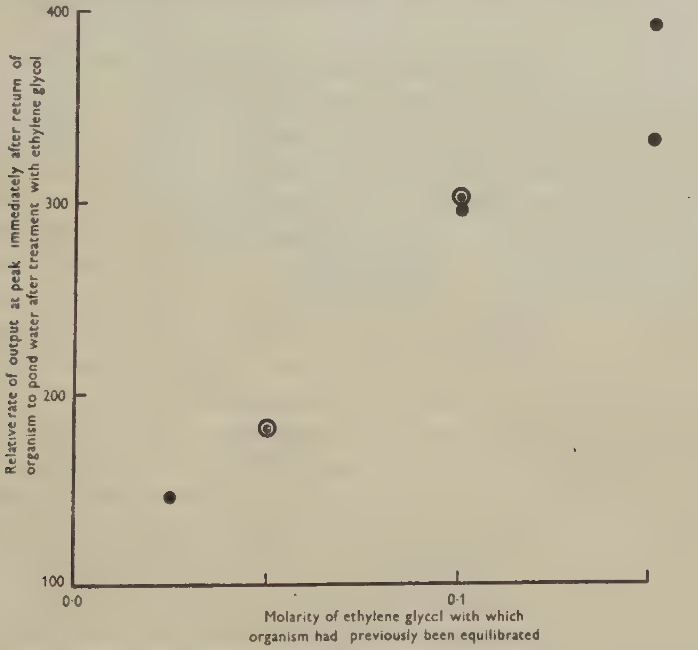

Fig. 6. The relation between the peak rate of vacuolar output of *C. aselli* on return to pond water and the concentration of ethylene glycol with which the organisms had previously been equilibrated. Experiments in which there was a detectable swelling of the body (after 0·5 and 0·25 M-ethylene glycol) have not been included. An outer circle indicates a coincident point.

The organisms appeared remarkably normal and healthy during the experiments. In those experiments in which there was a temporary strong shrinkage or swelling of the body, there was also at those times a stoppage of the cilia. Otherwise the cilia beat actively throughout the experiments.

DISCUSSION

Excess internal osmotic pressure of the fresh-water peritrich Carchesium aselli

No change could be detected in the body volume of *C. aselli* immersed in solutions of sucrose of strengths ranging up to about 0·04 M, whereas in 0·05 M solutions of sucrose there was slight shrinkage as previously recorded (Kitching, 1938). In the absence of other disturbing factors it would be concluded that the internal osmotic pressure of the organism exceeds that of pond water by that of a 0·04 M solution of non-electrolyte. It is therefore necessary to consider to what extent other disturbing factors may vitiate this estimate.

The action of the contractile vacuole tends to interfere with osmotic equilibrium between the organism and its environment; that indeed appears to be one of its functions. When the concentration of solutes in the external medium is low, the contractile vacuole operates at a high rate and presumably maintains the osmotic difference. The contractile vacuole may continue to function, although at a low rate of output, even when the concentration of the external medium is raised sufficiently to cause a shrinkage of the body; and under these conditions it may be expected to raise the internal osmotic pressure slightly above that of the experimental external medium. However, in the experiments described in this paper, the rate of vacuolar output of organisms in solutions of sucrose equal to or more concentrated than 0·04 M was quite low. For instance, in the experiments illustrated in Fig. 3, the rate of output in 0·04 M-sucrose was between 10 and 20% of the normal rate in pond water; and, other things being equal, the osmotic difference maintained by the action of the vacuole would only be 10–20% of the normal. The estimate of excess of internal osmotic pressure would therefore be subject to a correction of no more than this amount.

The results for rate of vacuolar output may also be used as evidence of the normal excess internal osmotic pressure of the organism. Provided that there is no change in any other relevant factor, such as the permeability of the body surface, and provided that a steady state of water balance has been attained, for all occasions on which there was no change of body volume the rate of vacuolar output should be proportional to the difference of osmotic pressure between the organism and the outside medium. The observations of vacuolar output, when plotted against concentration of sucrose in the external medium, should therefore lie on a straight line, the projection of which should intersect the axis representing concentration (Fig. 3), under the hypothetical condition of zero output, at a point representing the normal excess internal osmotic pressure of the organism. Owing to the variability of results no great reliance can be placed on experiments in which only one concentration of sucrose was used. The results of the two experiments in which more than one concentration of sucrose were used are shown in Fig. 3. From these the normal excess internal osmotic pressure would be estimated as that of 0·04 M-sucrose.

Estimates of the excess internal osmotic pressure obtained from vacuolar output at various concentrations of external medium are possibly liable to error owing to the uptake of water in food vacuoles. In the simplest and most extreme case, if all the water taken up in food vacuoles were evacuated through the contractile vacuole, an appropriate reduction could be made from each reading of vacuolar output. The uptake of water in food vacuoles for *C. aselli* in pond water at 15° C. does not normally exceed 15% of the vacuolar output, and owing to intermissions and to reduced frequency of formation of food vacuoles the absolute rate is no doubt much less in the higher concentrations of sucrose (0·03–0·05 M). A further complication is introduced by the fact that the rate of passage of water from the food vacuoles into the cytoplasm is also much less when there is sucrose in the external medium. If the rate of uptake of water in food vacuoles were always the same, and all the water were eliminated by the contractile vacuole, then the straight lines given in Fig. 3 should be displaced downwards by a distance representing this amount, i.e. 15% of the vacuolar output in pond water at 15° C.; this would reduce the estimate of normal excess internal osmotic pressure by about 0·006 M non-electrolyte. This represents an extreme value for the correction, and it is possible that no correction at all is required.

An indication of the normal excess internal osmotic pressure of *C. aselli* can also be obtained from the experiments in which organisms previously equilibrated with ethylene glycol were returned to pond water. From Fig. 6 it will be seen that the peak output, achieved immediately on transfer to pond water, rises by 100% of the normal vacuolar output for approximately every 0·05 M increase in concentration of the ethylene glycol. This result therefore suggests that the normal excess internal osmotic pressure is that of 0·05 M non-electrolyte. Escape of the ethylene glycol from the organism during the course of the readings might lead to a slight overestimate.

Estimates of the excess internal osmotic pressure of *C. aselli* in pond water at 15° C. are therefore as follows: from the effect of sucrose solutions on the body volume, 0·04 M non-electrolyte (possibly slightly too low); from the relation of vacuolar output to concentration of sucrose in the medium, 0·04 M non-electrolyte (possibly slightly too high); from the peak rate of output on return to pond water after equilibration with solutions of ethylene glycol, 0·05 M non-electrolyte (possibly slightly too high); and, for material from another source, from the effect of sucrose solutions on the body volume in the presence of cyanide (Kitching, 1938), 0·05 M non-electrolyte.

The general estimate of the internal osmotic pressure of *C. aselli* as that of 0·04–0·05 M non-electrolyte accords with experience of the effect of hypertonic solutions on this organism. It shrinks slightly in 0·05 M solutions of sucrose, and very strongly in 1·0 M solutions. If the excess internal osmotic pressure was only around 0·01 M, as suggested by Mast & Bowen (1944) for *Vorticella similis*, then it would shrink strongly both in 0·05 M solutions and in 1·0 M solutions. It is, however, interesting that Mast & Bowen have observed in *Vorticella* that the shrinkage of food vacuoles, which normally occurs in the first few minutes after uptake, is checked by the addition of sufficient sucrose—namely in 0·05 M concentration—to the medium. Apart from this, there is no reason why all peritrich ciliates should be considered as having the same internal osmotic pressure.

The effect of temperature on the uptake of water from the medium

The influence of temperature on the rate of osmotic uptake of water by *Carchesium aselli* from the external medium has a temperature coefficient of the order of 2·5–3·2. This is too great to be explained in terms of a direct effect of temperature on the osmotic pressure of the internal solution, since such an effect would only be proportional, approximately, to the change in absolute temperature ($Q_{10} < 1{\cdot}05$).

It is not possible to explain the results by postulating a change in the internal concentration of solutes. At 30° C. *C. aselli* also shrinks in solutions of sucrose at concentrations of 0·05 M-sucrose and upwards, so that the internal concentration of solutes cannot be materially greater than at 15° C. An increase of several-fold would be necessary to account for the observed change in the turnover of water.

The experiments on the osmotic swelling of *Vorticella marina* indicate a considerable effect of temperature on the initial rate of swelling. This may reasonably be interpreted as an effect on the permeability of the membrane through which the water diffuses. The figures suggest a Q_{10} of the order required, but they cannot be regarded as more than an approximate indication. However, the general conclusion finds support in the observations of Lucké, Hartline & McCutcheon (1931) on the eggs of *Arbacia*, and of Delf (1916) on onion leaf and dandelion scape.

It is therefore reasonable to conclude that the action of temperature on the uptake of water of a fresh-water peritrich, postulated in explanation of the ability of the organism to maintain its body volume in spite of great changes in vacuolar output, operates through an effect on permeability of the body surface, or part of it, to water. Change of permeability would include any effects on the diffusibility of water and on the properties of the membrane. The effect of temperature on the viscosity of water, as pointed out by Lucké & McCutcheon (1932) for echinoderm eggs, is not sufficient by itself to account for the results obtained. It has, however, been suggested (Davson & Danielli, 1943, p. 315) that the rate of passage of water through the living membrane is limited by the viscosity of the lipoid constituent, and it seems possible that the effect of temperature on the viscosity of the lipoid might be considerable. Perhaps, however, temperature has other profound effects on the structure of the membrane.

This effect of temperature on water exchange may have fairly widespread application among fresh-water animals. For instance, it has been shown that the rate of passage of water through frog's skin rises considerably with increase of temperature (Hevesy, Hofer & Krogh, 1935), and so does the urinary output (Krause, 1928). It may prove of some ecological interest that temperature can influence the osmotic stress to which an organism is subjected, and so may determine the osmoregulatory activity which that organism must maintain.

The activity of the contractile vacuole in relation to the osmotic stress imposed on the organism

Under the influence of temperature the rate of output of the contractile vacuole may be varied from about 20% at 0° C. to some 400–500% at 30° C. in relation to its normal level at 15° C., without detectable change in the body volume. It is therefore reasonable to suppose that in the case of changes of temperature the contractile vacuole responds primarily to the changed osmotic stress imposed upon the organism, rather than to the temperature directly, just as it must do in the case of the treatment with sucrose or ethylene glycol. This does not imply that the sensitivity of the mechanism may not be affected by temperature—that is to be expected in some measure—but osmotic stress by itself appears to be capable of eliciting the necessary response.

Presumably the osmotic stress imposed upon a *Carchesium* must influence vacuolar activity through an effect exerted on the cytoplasm. The most likely means is by a change in the water content of the cytoplasm, perhaps in relation to the proteins, although it is not possible to exclude from consideration changes in the concentration of other solutes. It is therefore reasonable to postulate that the contractile vacuole is sensitive to changes in the state of hydration of the cytoplasm, whether these arise through changes of temperature acting on the permeability of the membrane through which water enters the body or through changes in the concentration of solutes in the external medium. The magnitude of the hypothetical change in body volume necessary to cause a given change in rate of vacuolar output remains unknown in the case of *C. aselli*. It should be pointed out that a change of body volume by less than 10% probably would not be detected. In the case of certain marine peritrich ciliates it is known (Kitching, 1934) that the contractile vacuole is relatively insensitive to changes of concentration of the external medium, and so of body volume, over the range 100 to 50% sea water. Only when the external medium has been very considerably diluted, and the body has already swollen considerably, do further small changes make a substantial difference to the rate of vacuolar output. In the fresh-water peritrichs, however, it seems that the organism is already very sensitive, so that even a small change in hydration provokes a response sufficient to maintain the body volume constant within fairly narrow limits.

SUMMARY

1. On transfer from sea water to dilute sea water, the marine peritrich ciliate *Vorticella marina* swells more rapidly at higher temperatures.

2. It is concluded that the permeability of the surface of *V. marina* to water is influenced by temperature, with a Q_{10} of very roughly 2·5–3·2.

3. The body volume of the fresh-water peritrich ciliate *Carchesium aselli* is maintained approximately constant when the organism is transferred to solutions of sucrose of concentrations up to about 0·04 M; in higher concentrations the organism shrinks.

4. The rate of output of the contractile vacuole of *C. aselli* decreases with increasing concentrations of sucrose in the external medium; the rate of output is very low in 0·05 M-sucrose.

5. From a consideration of the effects of sucrose solutions on the body volume and on the rate of vacuolar output it is concluded that the initial osmotic pressure of *C. aselli* normally exceeds that of the external pond water by about 0·04–0·05 M non-electrolyte.

6. The internal osmotic pressure of *C. aselli* is not materially increased by increase of temperature.

7. It is concluded that the increase in rate of vacuolar output, which accompanies increase of temperature, counterbalances an increased rate of osmotic uptake of water from the external medium, and that this increased rate of uptake is due to an effect of temperature on the permeability of the surface through which the water enters.

8. The rate of vacuolar output is temporarily much increased when *C. aselli*, which has been equilibrated in solutions of ethylene glycol, is returned to pond water.

9. It is suggested that the temperature and the osmotic pressure of the external solution largely determine the osmotic stress which is imposed on the organism, and that they thus influence the state of hydration of the protoplasm; in turn this may be supposed to determine the activity of the contractile vacuole.

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